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STUDIES ON THE NATURE OF RESISTANCE OF *STREPTOCOCCUS FAECALIS* TO FOLIC ACID ANTAGONISTS*

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Abstract—Mutants of *Streptococcus faecalis* were selected at different levels of amethopterin ("methotrexate") in a medium containing pteroylglutamic acid (PGA). An increased capacity to reduce pteroylglutamic acid to the tetrahydro-level was a consistent characteristic of each of the resistant strains. The amount of aminopterin bound by the sensitive and resistant variants did not differ significantly. One highly resistant strain, which had been maintained for 2 years as a stock culture, took up much greater amounts of PGA and aminopterin than did the sensitive parent strain or the newly-selected resistant strains. This example of the concomitant occurrence of increased uptake and decreased effectiveness of a drug demonstrates the need for methods to study specific and non-specific binding in relation to drug resistance. An eighty-fold higher concentration of amethopterin relative to 4-amino-4-deoxy-^{10}N-methylpteroic acid, was required to obtain comparable degrees of inhibition of the growth of this amethopterin-resistant strain. Similarly, an eighty-fold higher concentration of amethopterin was required to inhibit the conversion of PGA to folinic acid by the intact cells; however, the soluble enzyme in cell-free filtrates was equally sensitive to inhibition by the two antagonists. Additional studies are needed to explain the mechanisms by which the access of the inhibitors to the sensitive enzyme system is limited in the drug-resistant cells.

DETAILED examination of individual characteristics associated with drug resistance is desirable whenever practical methods can be applied. Resistance of *Streptococcus facealis* to amethopterin ("methotrexate"; 4-amino-4-deoxy-^{10}*N*-methylpteroylglutamic acid) has been used as one model for such studies.

Most instances of drug resistance probably result from the cumulative influence of several factors. In amethopterin-resistant strains of *S. faecalis*, changes observed include an increased capacity to metabolize pteroylglutamic acid,[1, 2] altered uptake of the drug,[3, 4] altered nutritional requirements[5, 6] and decreased access of the drug to a sensitive enzyme system.[7] When the stepwise selection of resistant strains progresses slowly or the cultures are transferred repeatedly for prolonged periods, the opportunity for multiple changes to occur may be increased. In the study of individual characteristics that are associated with resistance, it seems preferable to use resistant

*This investigation is included as part of a thesis submitted by Aaron H. Anton for the degree of Doctor of Philosophy.

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cells which are removed from the sensitive parent population by a minimum number of generations. Thus, in this investigation, newly selected variants of *S. faecalis* of differing levels of resistance to amethopterin were studied primarily with respect to their capacity to bind the drug and to utilize the corresponding metabolite, pteroylglutamic acid (folic acid), as indicated by the formation of 5-formyl-5:6:7:8-tetrahydropteroylglutamic acid (folinic acid).

MATERIALS AND METHODS

Selection of drug resistant cultures. Variants of *S. faecalis* (ATCC strain 8043) with differing degrees of resistance to amethopterin were developed by exposing a culture derived from a single colony of the sensitive strain to exponentially increasing concentrations of amethopterin in a folic acid-assay medium[8, 9] containing PGA (1 mμg/ml). Resistant cultures were selected at each tenfold increase in the concentration of the antagonist. The variants of low resistance were selected after a few daily transfers and were then maintained on solid medium, whereas growth at the highest concentration of the drug occurred only after some forty daily transfers. Cultures were selected which varied in their exposure to concentrations of the antagonist ranging from 1 mμg to 100,000 mμg/ml of medium. To determine whether the resistance of the selected strains was a stable characteristic, the cultures were transferred daily in the absence of amethopterin. After the fifth such transfer, each strain was still able to grow at that concentration of amethopterin which was used initially for its selection. Each resistant strain was maintained in media containing the same amount of amethopterin as that used for the initial selection. A resistant culture, designated *S. faecalis/A*, which grows well in the presence of 100 μg/ml of amethopterin, had been selected 2 years previously under similar conditions, and had been maintained in media containing this concentration of the drug.

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Measurement of degree of resistance. Each culture was grown for 24 hr in the specified medium. The organisms were harvested by centrifugation, washed twice with a solution of sodium chloride (0·9%) (subsequently referred to as "saline") and diluted with the same solution to give a reading of 10 units on a Klett–Summerson photoelectric colorimeter, using a red filter (no. 66). One drop of this dilute suspension of bacterial cells was then inoculated aseptically into sterile medium (10 ml) containing varying amounts of the inhibitor and a sufficient amount of pteroylglutamic acid for maximum growth (1·0 mμg/ml). After incubation for 20 hr at 37 °C, the turbidity of each culture was measured in a Klett–Summerson colorimeter.

Preparation of cells and extracts for incubation. The amethopterin-resistant strains of *S. faecalis* and the sensitive parent strain were grown for 16 hr in a medium[9] containing PGA (2 mμg/ml). The cells were harvested by centrifugation and washed once with cold saline. The dry weight of the cells in each preparation was determined. The cells were suspended in phosphate buffer (0·2 M; pH 6·4) and were added to the respective incubation vessels.

A saline suspension of the washed cells (5%) was subjected to sonication (Raytheon Model DF 101; 30 min) and then centrifuged at 5000 *g* to remove the cell debris. The protein content of the clear supernatant solution was determined by the differential spectrophotometric method of Kalckar.[10]

Incubation procedures. The incubations were performed at 37° under flowing nitrogen in 20 ml beakers which were shaken constantly. For the measurement of "folinic acid" formation, the incubation mixture contained cells (4 mg, dry weight), PGA (0·22 μmoles), glucose (40 μmoles), sodium formate (20 μmoles), sodium ascorbate (20 μmoles) and phosphate buffer (0·2 M, pH 6·4) in a total volume of 2·0 ml. Incubations with the cell-free extract (3 ml of an extract containing 3 mg protein per ml) required, in addition to the above ingredients, ATP (7·5 μmoles), DPN (9·5 μmoles) and $MgCl_2$ (25 μmoles) in a total volume of 5 ml.[7] After incubation for 1 hr, the samples were heated immediately at 120° for 20 min and were then centrifuged. The supernatant fluid was assayed with *Pediococcus cerevisiae* 8081 using the medium described by Sauberlich[11]. This procedure gives a satisfactory measure of the reduction of PGA even though several steps are involved in the formation of folinic acid. The enzymic reduction and formylation of PGA in this sytem yields a heat-labile ^{5}N : ^{10}N-methylene derivative of tetrahydro-PGA which can be converted upon heating in the presence of a reducing agent to a stable form, folinic acid or 5-formyl-tetrahydro-PGA.[12–15]

Measurement of drug uptake. Aminopterin was used in these experiments, since similar hydrolysis of amethopterin yields ^{10}N-methyl-PGA, which cannot easily be measured microbially. The analogue was purified by ion exchange chromatography, using Dowex-1[16] and contained 0·06 per cent of contaminating growth-promoting substances, as measured by a microbial assay.[2] Alkaline hydrolysis of aminopterin converts this antagonist quantitatively to pteroylglutamic acid.[17] This procedure, combined with the microbial determination of folic acid, provided a sensitive, specific determination of the amount of aminopterin present in the cells. Mixtures containing cells (25 mg, dry weight), PGA or aminopterin (0·11 μmoles), glucose (50 μmoles) and phosphate buffer (0·2 M; pH 6·4) in a total volume of 5 ml were incubated as described above. After the incubation, those samples in which the uptake of PGA or aminopterin was to be determined were washed three times with ice-cold saline, then hydrolysed in 0·5 N NaOH (120 °C for 30 min), neutralized and finally assayed for folic acid activity by using *S. faecalis* 8043.[9]

EXPERIMENTAL AND RESULTS

Relationship between amethopterin resistance and growth in the presence of thymine, folic acid or folinic acid. S. faecalis 8043 responds equally well to either folic acid or folinic acid and the requirement for either of these forms of the vitamin can be obviated by the presence of thymine or thymidine in a medium which contains purines.[18, 19] Strains of *S. faecalis* were selected which could grow at concentrations of amethopterin ranging from 1 to 100,000 mμg/ml in media containing (1) PGA, 1·0 mμg/ml, or (2) 5-formyl-5:6:7:8-tetrahydro-PGA, 1·0 mμg/ml, or (3) thymine, 6·3 μg/ml. The level of resistance of these strains was then determined in the manner described, in each case the test medium containing PGA being used. Growth in the presence of even high levels of amethopterin was not accompanied by the selection of resistant strains when the medium contained thymine. Strains selected in the presence of folinic acid were somewhat less resistant than those selected in the presence of folic acid when tested under these conditions (Fig. 1). The level of resistance of strains selected in the medium containing folic acid was related to the final concentration of the drug used in each case during the selection.

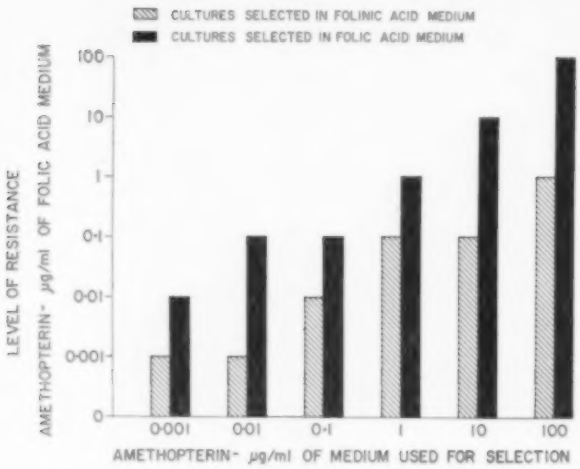

FIG. 1. Relationship of medium used for the selection of cultures to the level of resistance.

Relationship between degree of resistance and capacity to form a competing metabolite. The variants of *S. faecalis* with differing degrees of resistance to amethopterin (selected in the medium containing PGA) were compared with respect to their ability to form "folinic acid" from added PGA using intact cells and cell-free extracts of each strain (Table 1). The data indicate that the formation of folinic acid by the intact cells and the cell-free extracts was considerably greater in the resistant cells and was highest with the most resistant cultures. Also, the amount of antagonist needed to inhibit folinic acid formation by 50 per cent in the intact cells and cell-free extracts increased in a manner related to the degree of resistance (Table 1).

TABLE 1. SYNTHESIS OF FOLINIC ACID BY AMETHOPTERIN-RESISTANT STRAINS OF *Streptococcus faecalis*

Amethopterin tolerated by resistant strains during selection (μg/ml)	"Folinic acid" formed from PGA during incubation		Amethopterin conc. required for inhibition of "folinic acid" formation by 50 per cent	
	Intact cells (mμg/mg of cells, dry wt.)	Extract (mμg/mg of protein)	Intact cells (mμg/ml)	Extract (mμg/ml)
0	40	1	100	4
0·001	285	6	400	7
0·01	190	—	—	—
0·1	415	—	—	—
1	350	28	4000	10
10	880	—	—	—
100	910	120	20,000	25

Procedures were those described under Methods.

Uptake of aminopterin by different strains of S. faecalis. The uptake of the antagonist by the newly selected amethopterin-resistant strains of *S. faecalis* was not significantly different from that by the sensitive strain. However, it was found that *S. faecalis/A* consistently took up much greater amounts of aminopterin and of PGA than did the

sensitive strain (Table 2). The amounts of PGA and aminopterin taken up by the resistant organism were similar. It appears that in this instance the antagonist is bound in some manner that renders it innocuous.

TABLE 2. UPTAKE OF PTEROYLGLUTAMIC ACID AND AMINOPTERIN BY SENSITIVE AND RESISTANT STRAINS OF *Streptococcus faecalis*

Compound	Incubation time (min)	Amount recovered from washed cells	
		Sensitive strain *S. faecalis* 8043 (μg/g cells, wet wt.)	Resistant strain *S. faecalis/A* (μg/g cells, wet wt.)
Pteroylglutamic acid (μg/ml)			
—	—	0·18	0·18
10	15	4·12	23·4
10	30	5·76	36·0
10	60	8·65	52·0
Aminopterin (μg/ml)			
—	—	0·29	0·18
10	15	0·54	32·4
10	30	0·77	40·8
10	60	2·16	56·0

Procedures were those described under Methods.

Resistant cells (*S. faecalis/A*) which had been incubated with aminopterin were fragmented in the Raytheon sonic oscillator. A compound was found in the cell extracts which inhibited the growth of the sensitive strain of *S. faecalis* and had the same R_f value (0·24 with descending phosphate buffer; 0·1 M; pH 6·0) on paper chromatograms as has a sample of highly purified aminopterin. Since any capacity to inactivate aminopterin could be related to the ability of the resistant cells to grow in the presence of this drug, the recovery of aminopterin was estimated in two ways. The concentration of aminopterin was measured in duplicate preparations, before and after incubation, by measurement of its ability to inhibit the growth of the sensitive strain in liquid medium. Aminopterin was readily recovered from the cells by autoclaving at 15 lb/in^2 pressure for 10 min. Also, alkaline hydrolysis of duplicate preparations before and after incubation was followed by assay of the folic acid activity of the solutions. No evidence was obtained by these procedures that aminopterin was altered or inactivated during the incubation with these resistant cells.

Relationship between molecular structure and inhibitory potency. The compound 4-amino-4-deoxy-^{10}N-methylpteroic acid duplicates the structure of amethopterin except that it lacks the glutamic acid portion of the latter compound (Fig. 2). This pteroic acid analogue was only one-twelfth as effective as amethopterin in inhibiting by 50 per cent the growth of *S. faecalis* 8043. Since complete cross resistance between amethopterin and aminopterin was observed in the study of *S. faecalis/A*, it might be expected that similar cross resistance would occur between amethopterin and the pteroic acid analogue. It was found, however, that the growth of *S. faecalis/A* was eighty times more sensitive to inhibition by 4-amino-4-deoxy-^{10}N-methylpteroic acid than by amethopterin; inhibition of the growth of *S. faecalis/A* by 50 per cent occurred

at a concentration of the former compound of $1{\cdot}0 \times 10^{-5}$ M, whereas a similar degree of inhibition by amethopterin required a concentration of 8×10^{-4} M.

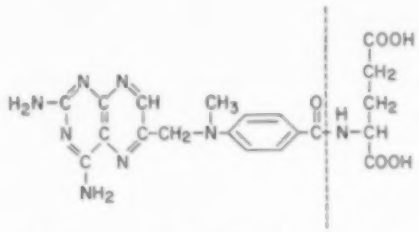

FIG. 2. Structural relationship between amethopterin and a pteroic acid analogue. 4-Amino-4-deoxy-^{10}N-methylpteroic acid duplicates the structure of amethopterin, with the exception of the glutamic acid-substituent.

The effectiveness of these two compounds in inhibiting the formation of folinic acid from added PGA by cell suspensions of the resistant organism was compared (Fig. 3). The data indicate that the pteroic acid analogue is nearly one hundred times

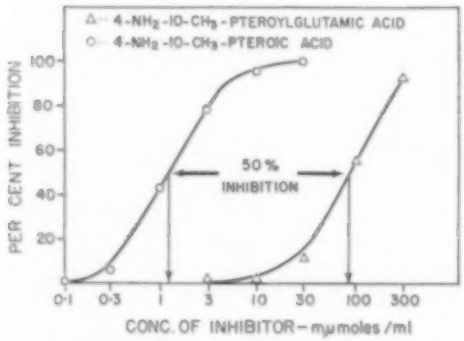

FIG. 3. Relative effectiveness of two similar analogues as inhibitors of folinic acid formation by cell suspensions of *S. faecalis/A*.

more effective than amethopterin in this respect. The parallelism of the two curves suggests that the inhibitory action of the two compounds was being exerted in a similar manner. When cell-free extracts of *S. faecalis/A* were used in a similar experiment, the formation of folinic acid was inhibited to the same extent by similar concentrations (2×10^{-7} M) of the two compounds.

DISCUSSION

A consistent characteristic of all of the newly selected resistant mutants was an increased capacity to metabolize PGA. Although there was not a close correlation between the magnitude of this change and the degree of resistance, the greatest amount of folinic acid was formed from PGA by the highly resistant cells. These findings are in agreement with earlier observations of an increased formation of folinic acid in amethopterin-resistant strains of *S. faecalis*.[1, 2] A similar alteration occurred in a sulphonamide-resistant strain of *Staphylococcus aureus* which formed more "folic acid"

than the sensitive parent strain when incubated with *p*-aminobenzoic acid.[20] Resistance to amethopterin does not appear to involve the development by *Streptococcus faecalis* of any amethopterin-insensitive pathway for the activation of PGA, as measured by the formation of reduced derivatives with folinic acid activity. Although somewhat higher concentrations of amethopterin were required to inhibit this reaction in extracts of the highly resistant cells, the insensitivity of the folinic acid-forming system in intact cells to high concentrations of amethopterin is in striking contrast to the sensitivity of this reaction in cell-free extracts to low concentrations of the drug. Two major characteristics associated with amethopterin-resistance in this organism, selected under these conditions, appear to concern the more efficient use of the metabolite, PGA, and the development of some means by which a sensitive enzyme system is protected from contact with the drug.

It is apparent that the composition of a medium can influence the level of resistance of cultures capable of growth in it. The degree of resistance of cultures selected in the presence of PGA was related to the concentration of the drug in the selecting medium. The level of resistance of cultures selected in the presence of 5-formyl-5:6:7:8-tetrahydro-PGA was considerably lower than the level of the drug in the selecting medium; however, a graded increase in resistance was observed. In the medium containing thymine, the organism grew readily at all concentrations of the inhibitor, and no selection of resistant cells occurred. The outgrowth of resistant variants would not be expected to occur unless the growth were limited by the concentration of thymine. Of particular interest in this regard are the observations that the presence of thymine in a *purine-free* medium containing folic acid reduced the lag-phase in the outgrowth of *S. faecalis* cells resistant to aminopterin[21, 22], and that thymine-requiring variants of *L. casei* were selected during the development of resistance to amethopterin[23] and pyrimethamine.[24] It would be expected, therefore, that in crude media or in a natural environment the availability either of metabolites produced by folic acid-dependent reactions or of folinic acid-like forms of the vitamin would condition both the ability of cells to grow in the presence of the drug and the emergence of a drug-resistant population.

The amount of aminopterin bound by different variants during incubation with cell suspensions was measured by a sensitive microbial determination of the PGA formed from aminopterin by alkaline hydrolysis. There was no consistent difference in the amount of aminopterin bound by the newly-selected resistant strains that could be related to the degree of resistance. Similar studies by Johnson and coworkers[4] based on the uptake of aminopterin-2-^{14}C indicated that other resistant strains of *S. faecalis* took up slightly more of the drug than did the sensitive strain. Only with the highly-resistant strain, *S. faecalis/A*, which had been selected similarly and maintained for more than 2 years in a similar medium containing amethopterin (100 μg/ml), was there a remarkable uptake of aminopterin, as compared to the sensitive "wild" strain of *S. faecalis*. This resistant organism also took up much greater amounts of PGA than did the sensitive parent. The occurrence of an increased uptake of aminopterin by cells which are not inhibited by it clearly indicates that the drug can be bound in an inactive form. This characteristic may be accentuated in *S. faecalis/A*. Similar binding of some fraction of the drug which penetrates the cell also may occur with other resistant variants. Even in the absence of any change in the total amount of the drug taken up, any decrease in the functionally effective proportion of

the antagonist taken up could be of real significance to the development of resistance.

Insensitivity to a drug, as the result of the development of a *decreased* capacity to "bind" the agent or to convert it into a metabolically active form, can readily be related to the occurrence of resistance. This characteristic has been observed repeatedly in studies on the nature of resistance of trypanosomes to certain toxic agents and of microbial resistance to certain antibiotics.[25] A number of examples demonstrate that similar changes are associated with microbial resistance to certain antimetabolites, such as resistance of tubercle bacilli to isoniazid,[26] resistance of *L. casei* to 2 : 6-diaminopurine,[2] and resistance of *S. faecalis* to 6-azauracil.[28] *Increased* binding of drugs by resistant organisms is more difficult to relate satisfactorily to the development of resistance. The occurrence of this characteristic, however, is not uncommon. More penicillin was taken up by some resistant strains of *Micrococcus pyogenes* and *S. faecalis* than by sensitive strains.[29, 30] Similarly, sulphanilamide was bound in larger amounts by strains of *Enterococci* and *E. coli* that were resistant to this drug.[31] Resistance of *Mycobacterium tuberculosis* to *p*-aminosalicylic acid was associated with the uptake of much larger amounts of the ^{14}C-labelled drug.[32] Larger amounts of unchanged aminopterin were extracted from an aminopterin-resistant strain of *Enterococci* than from the parent strain after incubation with this drug.[33]

The ability of *S. faecalis/A* to take up more aminopterin than the sensitive strain is of particular interest because of a difference in the inhibitory activity of closely related structures. Although some degree of cross resistance did occur, 4-amino-4-deoxy-^{10}N-methylpteroic acid was approximately eighty times more effective than amethopterin in inhibiting folinic acid formation by cells of this amethopterin-resistant strain. These observations suggest that the glutamic acid portion of the amethopterin molecule may be involved in the binding of this antagonist in an inactive form or with specific changes in transport mechanisms that prevent the drug from reaching a sensitive enzyme system. Two other observations of different responses to related glutamyl structures may be noted: *p*-aminobenzoylglutamate cannot supply the *p*-aminobenzoate requirement of *Leuconostoc mesenteroides* P-60 or of a nutritional mutant of *E. coli*;[13] pteroyltriglutamate (pteroylglutamyl-γ-glutamyl-γ-glutamate) cannot replace for the mono-glutamate as a vitamin for *S. faecalis*, even though enzymes capable of reducing this triglutamate to the corresponding derivative of folinic acid occur within these cells.[34, 35]

The concomitant occurrence of increased uptake and decreased effectiveness of a drug emphasizes the need for the development of methods of determining that fraction of the total amount of drug taken up by a cell which is accounted for by "specific-binding", that is, binding associated with inhibition of an enzyme. Comparison of the effectiveness of amethopterin and a related pteroic acid analogue in these studies implies that resistance in *S. faecalis/A* is associated with a structurally specific binding of amethopterin which prevents its access to a sensitive enzyme. The possible occurrence of similar drug-binding deserves examination in other instances of drug resistance.

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A 1:2-DIHYDRO-*s*-TRIAZINE WITH MARKED ACTIVITY AGAINST CERTAIN PATHOGENIC BACTERIA AND SYNERGISTIC WITH A SULPHONAMIDE IN EXPERIMENTAL BACTERIAL INFECTIONS

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Abstract—A dihydro-*s*-triazine (1-(3′:5′-dichlorophenyl)-2:2-dimethyl-4:6-diamino-1:2-dihydro-*s*-triazine) is described which has marked selective activity *in vitro* and *in vivo* against certain pathogenic bacteria. The most susceptible bacteria were streptococci of Lancefield Groups A, C, E, F and G, a type 2 pneumococcus, and a strain of *Streptococcus salivarius*. This triazine was also highly synergistic with sulfamethoxypyridazine when both were given orally in experimental infections caused by *Streptococcus pyogenes* *Diplococcus pneumoniae* and *Streptococcus salivarius*. The implications of these observations are discussed.

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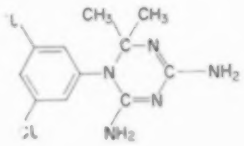
THIS is a partial and preliminary report on a study of a series of 1:2-dihydro-*s*-triazines concerned with their activity *in vitro* and *in vivo* against bacterial pathogens of medical importance. More specifically, this communication deals with representative experiments related to the antibacterial activities of 1-(3′:5′-dichlorophenyl)-2:2-dimethyl-4:6-diamino-1:2-dihydro-*s*-triazine, (here called "ABT-15251") the structure of which is given below:



In addition, a description is presented of the synergizing effects of this compound with the sulphonamide, sulphamethoxypyridazine (Midicel*), when given together in certain experimental bacterial infections in mice. Further studies on similar triazines, the synergism of this and other triazines with various sulphonamides, and additional details of antibacterial activity will be reported elsewhere.

Although other compounds related to the one reported here have been described with activity against certain protozoa[1, 2, 3, 4] and some strains of nonpathogenic bacteria,[5] no previous publication has described such activity against pathogenic bacteria, particularly *in vivo*. Indeed, there is indirect evidence, to be inferred from

*The trade name of Parke, Davis and Company for sulphamethoxypyridazine (3-sulphanilamido-6-methoxypyridazine) is "Midicel".

previous reports,[1, 6] that compounds of this type should not be effective *in vivo* against bacterial pathogens. However, it will be shown here that compound ABT-15251 exerts very marked selective inhibitory activity against certain of the pathogenic bacteria *in vitro*, and marked chemotherapeutic effects against infections in mice with some of these bacteria.

Of even greater importance, from the viewpoint of potential applications in the chemotherapy of bacterial infections, is the striking synergism effected by ABT-15251 when it is administered with sulphamethoxypyridazine in infected mice. The degree of this combined action resembles that previously reported by one of us (M.W.F.) of the synergistic combined action of human γ-globulin and chloramphenicol (Chloromycetin*) in experimental bacterial infections in mice.[7] Moreover, this triazine-sulphonamide synergism *in vivo* also has not been described in any previous publication with respect to pathogenic bacteria.

MATERIALS AND METHODS

Dihydrotriazine. ABT-15251 was first described by Crowther[8]; it was prepared for this work by the general method outlined in references 1 and 5.

In vitro. Stock solutions of ABT-15251 were autosterilized in 70% ethanol, and were then serially diluted in tubed trypticase soy broth (Baltimore Biological Laboratories) containing 10% bovine serum (v/v). Twofold decrements were prepared from an initial concentration of 100 μg/ml. Each 5 ml of medium was inoculated with 0·05ml of a 10^{-6} dilution of a fully grown broth culture of bacteria, these tubes were incubated for 18–24 hr at 37 °C, and then an estimate made of complete growth-inhibition end-points. Tests involving tubercle bacilli were performed in a like manner, with the exception that a defined basal medium was employed (consisting essentially of asparagine, glycerol and a few mineral salts) and incubation was for 7 days. All determinations *in vitro* were performed in duplicate and on separate occasions.

In vivo. Webster strain, Albino Swiss mice of either sex, weighing 18–22 g, were used throughout these studies. Acute and subacute infections were induced with the C203 strain of *Streptococcus pyogenes*, employing dosages of from 100 to 1000 LD_{50}. For the acute infection, 0·5 ml of a 10^{-5} dilution in broth of a 6 hr broth culture was administered intraperitoneally; this was uniformly lethal in untreated mice within 24 hr. The subacute infection was similarly prepared, with the exception that the mice were injected subcutaneously; without treatment the median survival time of mice so infected was approximately 5 days. Experiments with the SVI strain of *Diplococcus pneumoniae* involved the intraperitoneal injection of 0·5 ml of broth containing a 10^{-6} dilution of a 6 hr broth culture. This dose, 100 LD_{50}, effected a 90–100 per cent mortality in untreated mice within 3 days. Infection with the 04150 strain of *Streptococcus salivarius* was accomplished by the intraperitoneal injection of 0·5 ml of a 10^{-1} dilution of a 6 hr broth culture suspended in 5% hog gastric mucin, representing 100 LD_{50}. All untreated mice infected with this organism died within 48 hr.

All drug treatment was peroral, with various dose levels administered by intubation of 0·5 ml of a 5% acacia solution of each compound. In all instances, the treatment, which usually consisted of a single dose, was initiated within a few minutes after the infection was induced. Groups of from fifteen to twenty mice were employed per

*The trade name of Parke, Davis and Company for chloramphenicol is "Chloromycetin".

regimen, and all experiments were performed at least twice. Surviving mice were observed for 7–21 days after infection, depending on the acuteness of the infection.

In testing ABT-51251 alone, such dosage levels were selected from preliminary tests as would provide survival percentages ranging between 0 and 100. This permitted the estimation of the 50 per cent curative dose, employing the Miller–Tainter method.[9]

Table 1. *In vitro* antibacterial spectrum of ABT-15251

Organism	Strain	MIC* (μg/ml)
Aerobacter aerogenes	0126	>100·0
Brucella suis	H-1772	100·0
Corynebacterium diphtheriae	036	1·56
Diplococcus pneumoniae, type 1	SVI	100·0
Diplococcus pneumoniae, type 1	D-1	100·0
Diplococcus pneumoniae, type 2	D-2	0·012
Klebsiella pneumoniae	AD	100·0
Mycobacterium tuberculosis	H37Rv	50·0
Neisseria catarrhalis	03447	25·0
Neisseria meningitidis	B	50·0
Neisseria meningitidis	C	>100·0
Neisseria meningitidis	D	>100·0
Neisseria meningitidis	CDC	100·0
Pasteurella multocida	310	6·25
Proteus vulgaris	1810	100·0
Pseudomonas aeruginosa	28	50·0
Salmonella paratyphi	02156	50·0
Salmonella typhimurium	V31	50·0
Salmonella typhosa	02481	50·0
Shigella sonnei	04628	50·0
Staphylococcus aureus	Smith	100·0
Staphylococcus aureus	UC76	100·0
Staphylococcus aureus	04945	100·0
Staphylococcus aureus	04985	100·0
Streptococcus pyogenes, grp. A†	C203	0·02
Streptococcus pyogenes, grp. A	04772	0·1
Streptococcus pyogenes, grp. A	N-19	0·05
Streptococcus pyogenes, grp. A	GLN-55	0·02
Streptococcus pyogenes, grp. A	04715	3·13
Streptococcus pyogenes, grp. A	04364	3·13
Streptococcus pyogenes, grp. A	Rammelkamp	0·003
Streptococcus pyogenes, grp. A	Quinn	0·000002
Streptococcus agalactiae, grp. B	RI-090R	6·25
Streptococcus agalactiae, grp. B	IbH36B	100·0
Streptococcus sp., grp. C	Loosli	0·2
Streptococcus sp.., grp. C	674	0·012
Streptococcus sp., grp. C	CDC-SS-188	0·006
Streptococcus faecalis, grp. D	Viger	100·0
Streptococcus faecalis, grp. D	C-L	>100·0
Streptococcus faecalis, grp. D	CDC-SS-498	>100·0
Streptococcus faecalis, grp. D	MGH-1	>100·0
Streptococcus faecalis, grp. D	MGH-2	>100·0
Streptococcus faecalis, grp. D	MGH-3	>100·0
Streptococcus sp., grp. E	K129	0·098
Streptococcus sp., grp. E	K131	1·56
Streptococcus sp., grp. F	C628	0·024
Streptococcus sp., grp. F	B647	0·003
Streptococcus sp., grp. G	RI-D166B	0·006
Streptococcus sp., grp. G	RI-F68A	0·098
Streptococcus salivarius	04150	0·01

*Minimal inhibitory concentration.
†Designates Lancefield group.

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Combination therapy experiments with ABT-15251 and sulphamethoxypyridazine were designed so that minimally effective dose levels, of each compound alone, were employed. In these studies, simultaneous tests were conducted with the compounds separately and given as a mixture. The type of combination therapy experiments reported here involved the use of a constant dose of ABT-15251 given with varying dosages of sulphonamide. Similar effects were obtained in these infections when therapy was conducted with a fixed sulphonamide dose given with varying dosages of ABT-15251, but these will not be reported here.

RESULTS

The range of activity of ABT-15251 against various pathogenic bacteria *in vitro* is presented in Table 1. It is apparent that the most marked activity was shown against the six strains of *Streptococcus pyogenes*, one strain, "Quinn", being completely inhibited by as little as 0·000002 μg/ml. Good to marked inhibition was also observed with streptococcal species of Lancefield groups C, E, F and G, and with a strain of *Streptococcus salivarius*. Little or no activity was obtained against group D streptococci (six strains of *Streptococcus faecalis*), and a substantial difference in susceptibility occurred between the two strains of group B streptococci. An additional provocative observation was that a strain of type 2 pneumococcus was completely inhibited by 0·012 μg/ml, whereas two strains of type 1 required almost 10,000 times more compound for inhibition. Finally, less than 10 μg/ml was effective against *Corynebacterium diphtheriae* and *Pasteurella multocida*.

The nature of the activity of ABT-15251 given alone *in vivo* is illustrated by the data given in Tables 2, 3 and 4, involving infections with *Streptococcus pyogenes* (C203) and *Streptococcus salivarius* (04150). Although the experiments are not described here, it was observed that this compound was considerably more active when given subcutaneously in these infections. The fact that single doses of ABT-15251 were about equally effective in the acute and subacute streptococcal infections suggests that this compound either had a significant bactericidal action *in vivo* or persisted for prolonged periods in these mice.

TABLE 2. ACTIVITY OF ABT-15251 IN AN ACUTE INFECTION IN MICE INDUCED WITH *Streptococcus pyogenes* (C203)*

Dose of ABT-15251 (mg/kg)†	Survivors/total	Survivors %
0	0/20	0
0·625	1/20	5
1·25	2/20	10
2·5	3/20	15
5·0	9/20	45
10·0	14/20	70

50% curative dose: 6·0 ± 1 mg/kg.
*Induced intraperitoneally; median survival time untreated controls less 24 hr.
†Single oral dose given at time infection was induced.

Table 3. Activity of ABT-15251 in a subacute infection in mice induced with *Streptococcus pyogenes* (C203)*

Dose of ABT-15251 mg/kg†	Survivors/total‡	Survivors %
0	3/40	7·5
3·13	6/20	30·0
6·25	17/40	42·5
12·5	29/40	72·5
25·0	26/40	65·0
50·0	37/40	92·5
100·0	20/20	100·0

50% curative dose: 8·0 ± 2·3 mg/kg.
*Induced subcutaneously; median survival time of untreated controls approximately 120 hr.
†Single oral dose given at time infection was induced.
‡Data pooled from two experiments.

Table 4. Activity of ABT-15251 in an acute infection in mice induced with *Streptococcus salivarius* (04150)

Dose of ABT-15251 (mg/kg per day)*	Survivors/total	Survivors %
0	0/15	0
9·4	1/15	7
18·8	4/15	27
37·5	12/15	80
75·0	13/15	87

50% curative dose: 27·0 ± 5 mg/kg per day.
*Given in two divided doses, orally, on day infection was induced.

Table 5. Synergism between ABT-15251 and sulphamethoxypyridazine in an acute infection in mice induced with *Streptococcus pyogenes* (C203)

Dose of Sulphamethoxypyridazine (mg/kg)*		Dose of ABT-15251 (mg/kg)*	Survivors/total	Survivors (%)
0		0	0/20	0
0		0·5	2/20	10
3·13		0	0/20	0
6·25		0	0/20	0
12·5		0	0/20	0
25·0		0	2/20	10
3·13	+	0·5	9/20	45
6·25	+	0·5	12/20	60
12·5	+	0·5	15/20	75
25·0	+	0·5	17/20	85

*Single oral dose given at time infection was induced.

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TABLE 6. SYNERGISM BETWEEN ABT-15251 AND SULPHAMETHOXYPYRIDAZINE IN A SUBACUTE INFECTION IN MICE INDUCED WITH *Streptococcus pyogenes* (C203)

Dose of Sulphamethoxypyridazine (mg/kg per day)		Dose of ABT-15125 (mg/kg per day)	Survivors/total	Survivors (%)
0		0	0/20	0
0		0·25	0/20	0
3·13		0	0/20	0
6·25		0	0/20	0
12·5		0	2/20	10
25·0		0	6/20	20
3·13	+	0·25	8/20	40
6·25	+	0·25	10/20	50
12·5	+	0·25	13/20	65
25·0		0·25	16/20	80

*Given orally once daily for two consecutive days.

TABLE 7. SYNERGISM BETWEEN ABT-15251 AND SULPHAMETHOXYPYRIDAZINE IN AN ACUTE INFECTION IN MICE INDUCED WITH *Streptococcus salivarius* (04150)

Dose of Sulphamethoxypyridazine (mg/kg)*		Dose of ABT-15251 (mg/kg)*	Survivors/total	Survivors (%)
0		0	0/20	0
0		5	0/20	0
6·25		0	0/20	0
12·5		0	1/20	5
25·0		0	2/20	10
6·25	+	5	12/20	60
12·5	+	5	13/20	65
25·0	+	5	18/20	90

*Single oral dose given at time infection was induced.

TABLE 8. SYNERGISM BETWEEN ABT-15251 AND SULPHAMETHOXYPYRIDAZINE IN AN ACUTE INFECTION IN MICE INDUCED WITH *Diplococcus pneumoniae* (SVI)

Dose of Sulphamethoxypyridazine (mg/kg)*		Dose of ABT-15251 (mg/kg)*	Survivors/total	Survivors (%)
0		0	1/20	5
0		50	1/20	5
25		0	0/20	0
50		0	0/20	0
100		0	5/20	25
25	+	50	15/20	75
50	+	50	17/20	85
100	+	50	19/20	95

*Single oral dose given at time infection was induced.

The synergistic combined action of ABT-15251 and sulphamethoxypyridazine is clearly evident from the results given in Tables 5, 6, 7 and 8. There are a number of instances where dosages of either compound alone provided no activity whatsoever, but in which these same dosages given together caused the survival of 50 per cent or more of the mice. It is also apparent that in all instances minimally active dose levels of ABT-15251 alone, when given with sulphamethoxypyridazine, caused a many-fold increase in sulphonamide activity.

DISCUSSION

With regard to the pathogens tested herein, the high degree of selectivity of this triazine suggests that those organisms most susceptible, i.e. streptococci of groups A, C, E, F and G, may possess a vulnerable metabolic site that is distinctive and peculiar to these bacteria. Intraspecies differences, such as those exhibited by *Diplococcus pneumoniae*, are even more provocative, and merit further study.

Although it is not within the scope of this communication to discuss mechanisms of action, substantial evidence has been obtained that this and similar triazines do not act directly on known PABA–folic–folinic acid systems[10] in so far as a representative strain of *Streptococcus pyogenes* is concerned. These observations do not alone rule out the possibility that the triazine acts on the same metabolic sequence as sulphonamides. However, until some interrelationship is shown between triazines and sulphonamides with respect to sites of inhibition among these bacteria, there is no basis for explaining or predicting synergism.

In general, the susceptibility of an organism to either agent alone does not appear wholly to determine its response to combined treatment. For example, a strain of *Diplococcus pneumoniae* which was essentially insusceptible to the triazine both *in vitro* and *in vivo* was quite vulnerable to a synergistic combination of triazine and sulphonamide *in vivo*. As a rule, however, infections with bacteria which are highly senstive *in vitro* to the triazine alone will also respond synergistically *in vivo* to triazine-sulphonamide treatment. In this regard, the triazine-sulphonamide combination was synergistic *in vivo* against *Streptococcus pyogenes*, but such combined action was not observed *in vitro*. The use of a highly enriched culture medium may have served to suppress such effects.

The potential medical applications of a triazine-sulphonamide combination are quite evident from these experiments, particularly in streptococcal infections caused by Lancefield group A strains. The high degree of activity, effectiveness by oral administration, and specificity serve as desirable qualities. Additional studies related to chemotherapy are in progress and will be described when completed.

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CHEMICAL AND BIOLOGICAL STUDIES ON 1:2-DIHYDRO-*s*-TRIAZINES—XIII

INHIBITION OF RESPONSE TO *p*-AMINOBENZOIC ACID AND MECHANISM OF ACTION IN *LACTOBACILLUS ARABINOSUS* BIOASSAY SYSTEMS*

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Abstract—A series of 1:2-dihydro-*s*-triazines has been studied in *Lactobacillus arabinosus* 17–5 (ATCC no. 8014)–*p*-aminobenzoic acid bioassay systems. Differences in biological activity within the series could be correlated with certain variations in structure and substitution in the molecule. Maximum activity was observed both with 2:2-dimethyl substitution in the triazine ring together with 3'-halogen substitution in the phenyl ring, and with *n*-hexyl to *n*-tridecyl substitution at the 2-position of the triazine ring, irrespective of *p*- or *m*-phenyl substitution.

The active derivatives exhibited synergism with both sulphadiazine and aminopterin. Similarly, synergism between sulphadiazine and aminopterin was observed in this bioassay system. Inhibition analyses indicate that these three classes of inhibitor differ with respect to loci of activity.

In conjunction with previous evidence, the data suggest that in this *L. arabinosus*—PABA system, the 1:2-dihydro-*s*-triazines interfere with a diphosphopyridine nucleotide-mediated reduction of the pteroylglutamic acid (or pteroylglutamic acid-like) intermediate synthesized from *p*-aminobenzoic acid in the conversion of *p*-aminobenzoic acid to structures exhibiting citrovorum factor activity.

INTRODUCTION

Previous reports from these laboratories have described the synthesis[1–3] and the microbiological[4–8] and anti-tumour[9, 10] activity of a series of 1:2-dihydro-*s*-triazines (D·HCl's). Various derivatives of this series also have been shown to exhibit significant biological activity in experimental *Plasmodium*[1, 11] and *Eimeria*[12] infections, mammalian liver[13] and botanical systems,[14] experimental murine toxoplasmosis,[15] and in tissue culture.[16]

Previous studies have indicated that the primary mechanism of action of these compounds in microbiological systems is the inhibition of a diphosphopryidine nucleotide (DPN)-dependent reduction of pteroylglutamic acid (PGA) in the synthesis of structures exhibiting citrovorum factor (CF) activity.[17, 18] During the course of these investigations, it was observed that certain dihydrotriazines also inhibited the

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response of *Lactobacillus arabinosus* to *p*-aminobenzoic acid (PABA).[7] The present report is concerned with the results of studies originating from this observation.

METHODS

A bioassay system based upon the response of *Lactobacillus arabinosus* (17–5) no. 8014* to PABA[19, 20] was used in these experiments. Past experience indicated that the usual media prepared from hydrolysed casein were not entirely satisfactory, as judged by the results of routine replicate PABA titrations done over periods of several weeks. Inherent differences in the hydrolysis and purification by charcoal adsorption of crude caseins[21] have undoubtedly been a source of difficulty, since the response of *Lactobacillus arabinosus* to PABA has been reported to vary with the type of casein hydrolysate used.[22] Similarly, the presence of purine bases in the medium results in the growth of *Lactobacillus arabinosus* in the absence of PABA.[23]

The medium described by Koft *et al.*[24], with minor modifications, yields reproducible optical densities ($\times$ 10) of from 4·0 to 6·0 from minimal inocula of the stock strain of *Lactobacillus arabinosus* in the presence of PABA, and no growth in its absence. Bacto-vitamin-free casamino acids (Difco), 200 g/l., treated at pH 3·5 with 20 g/l. of Darco no. S-51 for 5 min at 25°C and adjusted to pH 5·8 with 40% NaOH,[21] were substituted for the usual casein hydrolysate at 0·5% final concentration. The L-tryptophan was treated similarly. The proper purification by adsorption of these ingredients is critical; too little treatment results in growth in the absence of PABA while over-adsorption results in growth failure, even in the presence of PABA or PGA. Since the addition of purine bases to the modified medium obviated the need for PABA, purines (and pyrimidines) were omitted. The glucose and phosphates were autoclaved as a separate solution, and the modified medium was otherwise identical with that described by Koft *et al.*[24]

All dilutions were made with double glass-distilled water in chemically clean glassware. The various solutions constituting the basal medium were autoclaved for 5 min at 15 lb/in² pressure, combined aseptically and dispensed in 10 ml volumes in optically standardized 22 $\times$ 175 mm tubes, which were closed with loose-fitting aluminium caps.

Crystalline PABA and other metabolites, the various D·HCl's, and the other inhibitors used were prepared in appropriate concentrations in sterile double glass-distilled water, and were added aseptically in volumes of 0·1 ml to the cooled, sterile medium to the desired final concentration.

Inocula were prepared daily from viable stock cultures of *Lactobacillus arabinosus* maintained on 5% horse blood agar plates. The surface colonies were emulsified in sterile, physiologic saline, and the suspension of bacteria was adjusted to an optical density of approximately 0·05 $\times$ 10; 0·1 ml of the suspension was added to each 10 ml tube of media. All titrations were incubated for 48 hr at 37 °C. Optical density was read directly in a Lumetron colorimeter adjusted so that medium blanks read zero vs. the 650 mμ filter.

The synthetic medium *C* described by Dunn *et al.*,[25] without purines, also was used for certain experiments on the growth response to PABA. It is of interest to note that unlike the results obtained by others,[26] L-methionine did not satisfy the PABA require-

*American type culture collection number.

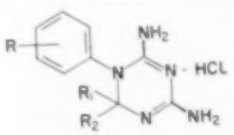
ment of the strain of *Lactobacillus arabinosus* used in these experiments. Apparently at least two varieties of *Lactobacillus arabinosus* 17–5 exist, a PABA-requiring and a PABA-independent strain, which are otherwise identical;[27, 28] however, it is evident that the strain used in the present studies possesses a clear-cut PABA requirement when cultured in an appropriate medium.

The D·HCl's and other inhibitors used were titrated from 1 mg/ml to the minimal inhibiting dose in the presence of 0·1, 0·01 or 0·001 μg of PABA per ml, the last concentration being the minimal one which supported maximal growth under the present experimental conditions. The 50 per cent minimal inhibiting dose[29] for excess and minimal concentrations of PABA was interpolated from semi-log plots of the data obtained from such titrations. Inhibition analyses[30] were done in systems containing 0·001 μg of PABA per ml, to which were added the minimal (or multiples of) concentrations of the respective D·HCl's that effected 50 per cent inhibition of growth, as determined by previous titration. Various metabolites were then titrated in these inhibited systems to determine their ability to reverse inhibition.

Structure–activity relationships

Halogen substitution. Chloro- or bromo-substitution at the 3′-position of the phenyl ring resulted in increased activity as compared with the unsubstituted phenyl derivatives (Table 1). Unlike the results obtained in *Streptococcus faecalis*–PGA bioassay systems,[4] di-halogen substitution did not result in a further increase in activity beyond that characteristic of the compounds bearing a halogen at the 3′-position of the phenyl ring, e.g. D-69·HCl and D-54·HCl, Table 1. Those derivatives with asymmetrical substitution at the 2-position of the triazine ring (Table 1) were generally less active than the corresponding 2:2-dimethyl-substituted compounds.

Table 1. Inhibitory activity of 1:2-dihydro-*s*-triazines in *p*-aminobenzoic acid bioassay systems



Compound no.	Substitution			50% inhibition index ($\times 10^{-2}$) *vs.* *Lactobacillus arabinosus*, PABA, 0·001 μg/ml
	R	R_1	R_2	
D-23·HCl	H	CH_3	CH_3	2·7
D-69·HCl	3′-Cl	CH_3	CH_3	0·2
D-91·HCl	3′-Br	CH_3	CH_3	0·25
D-20·HCl	4′-Cl	CH_3	CH_3	2·7
D-65·HCl	4′-Br	CH_3	CH_3	2·5
D-54·HCl	3′ : 4′-Cl_2	CH_3	CH_3	0·24
D-66·HCl	H	CH_3	C_2H_5	100+
D-114·HCl	3′-Cl	H	C_2H_5	3·5
D-130·HCl	3′-Br	H	C_2H_5	3·2
D-43·HCl	4′-Cl	CH_3	C_2H_5	21·0
D-75·HCl	3′ : 4′-Cl_2	CH_3	C_2H_5	2·8
D-67·HCl	3′ : 4′-Cl_2	H	CH_3	2·0

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Structural alterations resulting in loss of activity. The increased activity resulting from *m*-substitution in the phenyl ring is further emphasized by comparison with the corresponding *p*- and *o*-derivatives of the halogen-substituted series (Table 2). Substi-

TABLE 2. EFFECT OF PHENYL SUBSTITUTION ON ACTIVITY OF 1:2-DIHYDRO-2:2-DIMETHYL-*s*-TRIAZINES* IN *p*-AMINOBENZOIC ACID BIOASSAY SYSTEMS

Compound no.	Substitution*† R	50% inhibition index ($\times 10^{-3}$) *vs.* *Lactobacillus arabinosus*, PABA, 0·001 μg/ml
D-102·HCl	4′-F	3·0
D-103·HCl	2′-F	100·0+
D-83·HCl	4′-CH_3	1·8
D-93·HCl	3′-CH_3	0·3
D-38·HCl	2′-CH_3	100·0+
D-92·HCl	2′ : 6′-$(CH_3)_2$	100·0+
D-20·HCl	4′-Cl	2·7
D-69·HCl	3′-Cl	0·2
D-68·HCl	2′-Cl	15·0
D-96·HCl	4′-OCH_3	3·5
D-98·HCl	2′-OCH_3	100·0+
D-65·HCl	4′-Br	2·5
D-91·HCl	3′-Br	0·25
D-82·HCl	2′-Br	100·0+
D-100·HCl	4′-I	4·5
D-128·HCl	3′-I	0·52

*See Table 1 for structure.
†In this series, $R_1 = R_2 = CH_3$.

tution at the *o*-position of the phenyl ring or the presence of a large blocking group at the 2-position of the triazine ring resulted in a marked decrease in activity (Table 2), as observed in other bioassay systems.[4–7] Similarly, the anilinodihydrotriazines prepared by isomerization of the corresponding D·HCl derivatives[1, 2] were relatively inactive, as was the case in other bioassay systems.[4–7]

Effect of 2-(n-*alkyl*) *substitution.* As illustrated in Table 3, activity increases markedly as the number of carbon atoms in *n*-alkyl configuration at the 2-position of the triazine ring increases beyond *n*-butyl or *n*-pentyl. Maximum activity is obtained when the substituent at the 2-position is between *n*-hexyl and *n*-tridecyl, irrespective of substitution in the phenyl ring (Table 3). This increase in activity with increasing length of the carbon chain at the 2-position of the triazine ring differs from that observed with *Streptococcus faecalis* and *Pediococcus cerevisiae*[31] (formerly termed *Leuconostoc citrovorum*) only in that, in these latter bioassay systems, maximum activity was more sharply defined between the *n*-hexyl and *n*-decyl derivatives.

The inhibition induced by this series of compounds is essentially non-competitive with respect to PABA, as well as to PGA and its derivatives in this, as in other[4–7] bioassay systems.

Table 3. Effect of 2-(*n*-alkyl) substitution at 2-position of triazine ring on activity of 1:2-dihydro-*s*-triazines in *p*-aminobenzoic acid bioassay systems

Compound no.	Substitution*		50% inhibition index ($\times 10^{-3}$) *vs.* *Lactobacillus arabinosus*, PABA, 0·001 μg/ml
	R_1	R_2	
R = H			
D-133·HCl	H	C_2H_5	3·2
D-136·HCl	H	*n*-C_4H_9	3·8
D-134·HCl	H	*n*-C_5H_{11}	0·35
D-113·HCl	H	*n*-C_6H_{13}	0·35
D-111·HCl	H	*n*-$C_{11}H_{23}$	0·01
D-121·HCl	H	*n*-$C_{13}H_{27}$	0·03
D-112·HCl	H	*n*-$C_{17}H_{35}$	3.0
R = 3′-Br			
D-130·HCl	H	C_2H_5	3.2
D-131·HCl	H	*n*-C_5H_{11}	0·35
D-124·HCl	H	*n*-C_6H_{13}	0·3
D-122·HCl	H	*n*-$C_{11}H_{23}$	0·025
D-139·HCl	H	*n*-$C_{13}H_{27}$	0·03
R = 3′-Cl			
D-114·HCl	H	C_2H_5	3·5
D-126·HCl	H	*n*-C_5H_{11}	0·23
D-110·HCl	H	*n*-C_6H_{13}	0·23
D-109·HCl	H	*n*-$C_{11}H_{23}$	0·031
R = 4′-Cl			
D-45·HCl	H	CH_3	10·0
D-60·HCl	H	C_2H_5	12·1
D-48·HCl	H	*n*-C_3H_7	7·5
D-87·HCl	CH_3	*n*-C_3H_7	3.7
D-84·HCl	H	*n*-C_6H_{13}	2·5
D-127·HCl	H	*n*-C_9H_{19}	0·023
D-106·HCl	H	*n*-$C_{11}H_{23}$	0·016
R = 3′:4′-Cl_2			
D-67·HCl	H	CH_3	2·0
D-63·HCl	H	C_2H_5	3·2
D-76·HCl	H	*n*-C_3H_7	3·1
D-123·HCl	H	*n*-C_4H_9	2·9
D-78·HCl	H	*n*-C_5H_{11}	3·0
D-95·HCl	H	*n*-C_6H_{13}	2·8
D-117·HCl	H	*n*C_7H_{15}	0·3
D-118·HCl	H	*n*-C_8H_{17}	0·031
D-115·HCl	H	*n*-C_9H_{19}	0·029
D-116·HCl	H	*n*-$C_{10}H_{21}$	0·025
D-101·HCl	H	*n*-$C_{11}H_{23}$	0·035
D-108·HCl	H	*n*-$C_{13}H_{27}$	0·02

*See Table 1 for structure.

Mechanism of action

Reversal. The minimal concentrations of PGA and of certain of its derivatives which support maximal growth of *Lactobacillus arabinosus* under the present experimental conditions are compared with PABA in Table 4. The effect of the addition of these and other metabolites to systems inhibited by the D·HCl's is also illustrated in Table 4.

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TABLE 4. REVERSAL OF INHIBITION IN *Lactobacillus arabinosus*–*p*-AMINOBENZOIC ACID BIOASSAY SYSTEMS: COMPARISON OF 1:2-DIHYDRO *s*-TRIAZINES, SULPHADIAZINE AND AMINOPTERIN

Metabolite*	Minimal effective concentration for maximal growth (μg/ml)	Reversal of inhibition in the presence of PABA (0·001 μg/ml)			
		Conc. of reversing agent (μg/ml)	Inhibitor†		
			D-54·HCl (0·24 μg/ml)	Sulphadiazine (0·6–1·0 μg/ml)	Aminopterin (0·02–0·03 μg/ml)
7:8-Dihydropteroylglutamic acid	0·001	0·1	Complete‡	Complete‡	Complete‡
Natural citrovorum factor	0·001	0·005	Complete	Complete	Complete
Thymidine	0·1	0·1	Complete	Complete	Complete
Diphosphopyridine nucleotide	§	0·1	Complete	None	None
Nicotinic acid	§	1·0	Complete	None	None
Nicotinamide	§	1·0	Complete	None	None
p-Aminobenzoic acid	0·001	1·0–10·0	Partial**	Complete	Complete
Pteroylglutamic acid	0·1	10·0+	Partial	Complete	Complete
^{10}N-Formylpteroylglutamic acid	0·01	10·0+	Partial	Complete	Complete
Ascorbic acid	§	2500	Complete	Partial**††	None
Vitamin B_{12}	§	100	Complete	None	None

*Other growth factors without effect as reversing agents except purines, which eliminate PABA requirement of *L. arabinosus*.
†All inhibitors employed at the 50 per cent minimal inhibiting dose (50 per cent MID) and multiples thereof. Results similar to those reported with D-54·HCl obtained with several other dihydrotriazines at their 50 per cent MID and multiples thereof (cf. Tables 1–3).
‡Effective against as much as 4-10 × 50 per cent MID of inhibitor.
§Employed as reversing agent only; will not support growth in the absence of purines or the other metabolites listed.
**Effective against only 1-3 × 50 per cent MID of inhibitor.
††L-Cysteine exhibits similar activity; other biological reducing agents ineffective.

7:8-Dihydro-PGA,*[32] natural CF,†[33] thymidine or DPN‡ are more effective in the reversal of inhibition than is either PABA or unaltered PGA, even when the latter are provided in excess of the concentrations required for maximal growth in uninhibited systems. Similarly, ^{10}N-formyl-PGA†[34] is no more effective than unaltered PGA. Reversal by reduced, but not unaltered or formylated PGA, together with reversal by DPN or its precursors, suggests that in this system the D·HCl's interfere with the DPN-mediated reduction of PGA, as has been described in other microbiological systems.[8, 17, 18]

The effects of ascorbic acid and vitamin B_{12} (Table 4) also are similar to those observed in other microbiological systems inhibited by the D·HCl's.[8, 17, 18] The effectiveness of ascorbic acid in reversing inhibition is probably the result of physicochemical alteration of the redox potential, as appeared to be the case previously,[8, 17, 18] rather than a specific metabolite response. Neither ascorbic acid nor other biological reducing agents satisfy the nicotinic acid requirement of *Lactobacillus arabinosus*. The observation that vitamin B_{12} exhibits some activity, particularly when the medium contains either reduced PGA or CF, again suggests[8, 17, 18] an intimate relationship between the metabolism of vitamin B_{12} and either the synthesis or the utilization of structures exhibiting CF activity, or both. That DPN (or a suitable precursor) is essential to the cell for purposes other than the synthesis of CF is evident from the inability of reduced PGA or preformed CF to support the growth of *Lactobacillus arabinosus* in the absence of DPN.

Inhibition of *Hemophilus parainfluenzae* in the DPN-bioassay systems by the D·HCl's and reversal of inhibition by DPN have been reported.[17] This inhibition has been shown to be competitive over at least a 100-fold range of concentration of DPN.§ The results of other experiments with *Lactobacillus arabinosus*, wherein DPN and its precursors were titrated in the presence of 0·001 μg of PABA per ml, indicate a competitive relationship between the D·HCl's and DPN over at least a tenfold range of concentration, whereas the inhibition of nicotinic acid, nicotinamide, PABA and PGA is noncompetitive (Table 5). Inhibition of DPN by the D·HCl's in either bioassay system was readily reversed by excess DPN or its precursors, whereas inhibition induced by 4-APGA or sulpha in similar experiments was not so reversed (Table 4).

The possible argument that the adenine component of DPN might be released and utilized under the experimental conditions of *Lactobacillus arabinosus* assay is considered to be invalid. Reversal of D-54·HCl inhibition by DPN (0·1 μg/ml) (Table 4) cannot be due to liberated adenine inasmuch as there is insufficient purine present in that quantity of DPN to support growth, even if there were complete cleavage of the adenine component. In addition, neither DPN nor its precursors, nicotinic acid or

*Synthesized by one of us (E.J.M.), after O'Dell *et al.*[32]

†We wish to express our appreciation to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for generous samples of these compounds. Although none of the 4-hydroxypteridines used in the reversal studies was tested for chromatographic purity, each was shown to be 95-100 per cent pure by ultra-violet spectral analysis. This evidence, together with a careful analysis of the reversal data in Table 4, precludes erroneous conclusions due to the possible presence of small amounts of PABA or *p*-aminobenzoylglutamic acid in the pteridine samples used.

‡Pabst Laboratories, Milwaukee, Wisconsin, purity 96 per cent. Pabst TPN (purity 100 per cent) was but slightly effective (less than 10 per cent) in reversing D·HCl inhibition in these systems.

§In *Hemophilus parainfluenzae*–DPN dependent systems, full growth is obtained at concentrations of 0·001, 0·01 and 0·1 μg/ml of DPN. At these concentrations, for example, inhibition indices of 1000, 1000 and 900, respectively, were obtained with D-95·HCl. For experimental details, cf. ref. 17. These studies will be published in detail in another paper.

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nicotinamide, will support the growth of *Lactobacillus arabinosus* in the absence of purines, PABA, PGA or its derivatives (Table 4). Although purines obviate the requirement for PABA in the present bioassay system, they do not replace the specific requirement of *Lactobacillus arabinosus* for either nicotinic acid, nicotinamide or DPN, even in the presence of PABA, PGA or its derivatives. Furthermore, other assay media (e.g. Difco) designed specifically for the assay of nicotinic acid or its derivatives contain both purines and PABA. Neither excess adenine nor guanine reverses D·HCl inhibition in any of the microbiological systems studied in these laboratories,[4–8, 17] whereas D·HCl inhibition is readily reversed by nicotinic acid, nicotinamide or DPN in other microbiological systems[7, 8, 17, 18] as well as in *Lactobacillus arabinosus*–PABA assay systems (Table 4).

TABLE 5. FIFTY PER CENT INHIBITION INDICES FOR D-69·HCl* IN *Lactobacillus arabinosus*–PABA ASSAY SYSTEMS

Metabolite	Concentration (μg/ml)					
	0·001	0·01	0·1	1·0	10·0	100·0
DPN†			3·5	0·65	0·08§	0·08§
Nicotinic acid†	150	30§	2·9§	0·29§	0·02§	toxic
Nicotinamide†	150§	22§	2·5§	0·30§	0·032	toxic
PABA‡	180§	55§	31§	3·0§	0·6§	toxic
PGA‡	200§	63§	29§	4·2§	1·5§	toxic

*Similar results obtained with several other active D·HCl compounds (cf. Tables 1–3).

†In presence of 0·001 μg of PABA per ml. Does not support growth in absence of PABA, or PGA or its derivatives.

‡In presence of 2·0 μg of nicotinic acid per ml. Does not support growth in absence of nicotinic acid, nicotinamide or DPN.

§Maximum growth obtained at these concentrations of metabolite in absence of inhibitor.

Comparison with 4-APGA and sulpha. The pattern of reversal of 4-APGA differs from that of D·HCl inhibition, as in other bioassay systems.[5, 6, 17, 18] 4-APGA is an effective inhibitor in this *Lactobacillus arabinosus*–PABA bioassay system, 0·02–0·03 μg/ml inducing 50 per cent inhibition. Inhibition is completely reversed by PABA, PGA or its derivatives, adenine, guanine or thymidine, poorly by thymine, and not at all by various biological reducing agents, vitamin B_{12}, DPN or its precursors (Table 4).

Sulpha is a less potent inhibitor than 4-APGA, about 0·6–1·0 μg/ml being needed for 50 per cent inhibition. The pattern of reversal of sulpha inhibition resembles that of 4-APGA (Table 4), except that high concentrations of ascorbic acid or L-cysteine are partially effective. DPN and its precursors, however, were ineffective, in contrast to the reversal of the D·HCl's by these metabolites.

Considerable experimental evidence indicates that PABA serves as a precursor of a substance (or substances) exhibiting CF activity[35] which may differ in certain respects from natural CF,[33] and that PGA or PGA-like substances may be intermediate in the biosynthesis of CF activity by *Lactobacillus arabinosus*.[36, 37] Differences in the inhibitory effects of sulphanilamide and 4-APGA upon this biosynthesis have been reported by Mitbander and Sreenivasin,[35] who observed that sulphanilamide was the more effective inhibitor when PABA was the precursor, whereas 4-APGA was more

effective when PGA was the precursor of CF activity in resting cell preparations of *Lactobacillus arabinosus*. These differences in the effectiveness of sulpha and 4-APGA also have been observed in the present studies.

Similarly, sulpha and D·HCl inhibition differ when PABA and PGA are utilized as precursors of CF activity (Fig. 1). Sulpha is most effective against PABA, while the

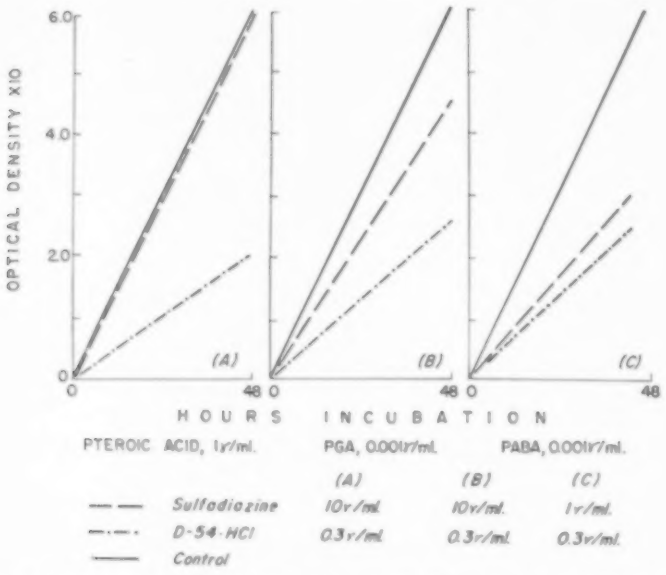

FIG. 1. Relative effectiveness of sulphadiazine and 1:2-dihydro-s-triazine (D-54·HCl)* *vs.* different precursors of citrovorum factor activity in *Lactobacillus arabinosus* systems.

*cf. Table 1 for structure.

D·HCl's are equally effective against PABA or PGA. This difference in relative activity is also observed when pteroic acid* is used as a precursor. The response of *Lactobacillus arabinosus* to pteroic acid is unaffected by sulphonamides,[38] while the D·HCl's are as active as when either PABA or PGA is the precursor (Fig. 1). Similar results have been obtained with certain other synthetic substances which exhibit PGA-like activity in appropriate microbiological systems.[39]

Synergism with sulpha and 4-APGA. Different loci of inhibition by sulpha and the D·HCl's are further evidenced by synergistic activity, as illustrated in Fig. 2 (A). In this method of graphic analysis,[40, 41] fractions of the 50 per cent minimal inhibiting concentration of one inhibitor are titrated in the presence of several different fractions of the 50 per cent minimal inhibiting concentration of the second inhibitor and the various fractional combinations required for 50 per cent inhibition determined. The concentrations so obtained are then plotted as fractional values of unity as represented by the 50 per cent minimal inhibiting concentration of each inhibitor on one axis. In the case of potentiation, the resulting figure is displaced to the left, the extent of deviation representing a measure of synergism, as illustrated in Fig. 2 (A). It is of

*See second footnote on p. 24.

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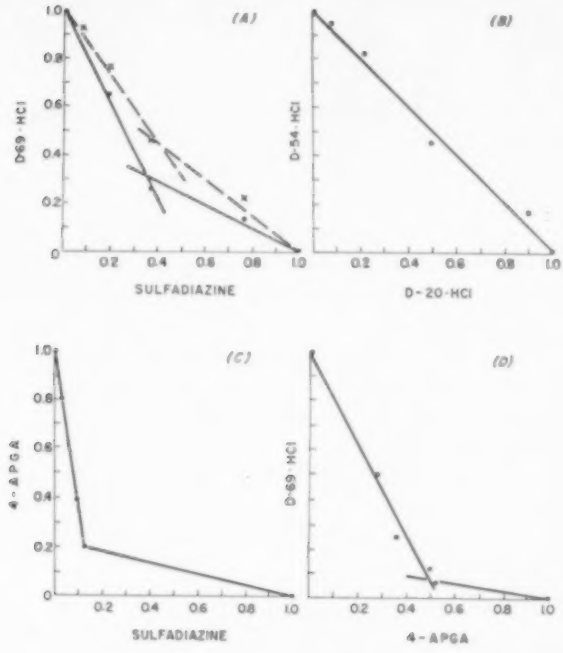
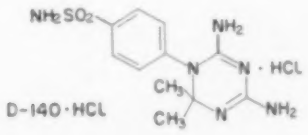


FIG. 2. Effect of combinations of inhibitors in *Lactobacillus arabinosus*—*p*-aminobenzoic acid systems (A) 1:2-dihydro-*s*-triazine (D-69·HCl)* and sulphadiazine (————); 1:2-dihydro-*s*-triazine (D-122·HCl)* and sulphadiazine (— — — —). (B) Two 1:2-dihydro-*s*-triazines (D-54·HCl, D-20·HCl)*. (C) 4-Aminopteroylglutamic acid and sulphadiazine. (D) 4-Aminopteroylglutamic acid and 1:2-dihydro-*s*-triazine (D-69·HCl)*.

**cf.* Table 1 for structures.

interest to note that despite the synergism exhibited by combinations of D·HCl and sulpha, a compound (D-140·HCl)* incorporating the structural features of sulphanilamide into the D·HCl molecule:



D-140·HCl

was inactive in *Lactobacillus arabinosus*–PABA systems. On the other hand, additive effects of combinations representing activity at a single locus result in a straight line with a slope of 45°, as illustrated in Fig. 2 (B). Similar analyses of combinations of sulpha with 4-APGA (Fig. 2 (C)) and D·HCl with 4-APGA (Fig. 2 (D)) also indicate

*The synthesis of this and the other previously undescribed dihydrotriazines considered here will be reported elsewhere.

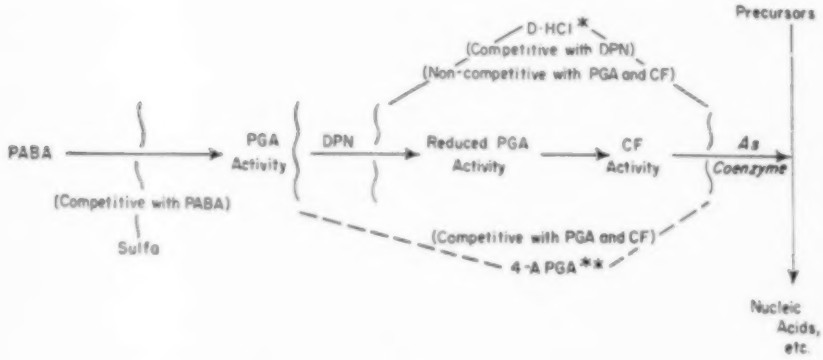
potentiation, and, accordingly, different loci of activity for 4-APGA, sulpha and D·HCl inhibition.

DISCUSSION

The results of inhibition analyses presented here indicate that in *Lactobacillus arabinosus*–PABA assay systems, the D·HCl's interfere with the biosynthesis of metabolic derivatives of PGA which are determinable as CF. The relatively poor reversal obtained with PGA or PABA, as compared with the effectiveness of reduced PGA, CF or DPN in reversing D·HCl inhibition, suggests that inhibition is the result of interference with a DPN-mediated reduction of PGA, or PGA-like structures synthesized from PABA, rather than of interference with the synthesis of the intermediate from PABA. The results presented in this communication and the accompanying paper[42] further support previous evidence that the primary mechanism of action of the D·HCl's in several microbiological systems[17, 18] appears to be interference with a DPN-mediated reduction concerned with PGA metabolism.

Conclusive evidence that the D·HCl's, in contrast to the analogues of PGA, block the reduction of PGA in certain microbiological systems has been presented.[5, 8, 17, 18] The competitive inhibition of DPN in *Lactobacillus arabinosus*–PABA assay systems is consistent with the inhibition of DPN by the D·HCl's in DPN-dependent *Hemophilus parainfluenzae* assay systems.*

The possible loci of D·HCl inhibition in this *Lactobacillus arabinosus*–PABA assay system are illustrated in Fig. 3. Although there is evidence that 4-APGA blocks



* D-HCls are relatively weak inhibitors of CF activity.

** 4-APGA blocks conversion of PGA activity to CF activity but not at DPN step.

FIG. 3. Postulated locus of 1:2-dihydro-*s*-triazine inhibition in conversion of *p*-aminobenzoic acid to citrovorum factor activity* by *Lactobacillus arabinosus*.
*All metabolic derivatives of PGA determinable as CF.

a reductive step in the conversion of PGA to CF activity in avian and mammalian liver systems *in vitro*,[43–46] there is no indication in this *Lactobacillus arabinosus*–PABA assay system (or in the other microbiological systems studied[4–6, 17, 18]) that 4-APGA inhibits a DPN-linked reductive step. Accordingly, the locus of inhibition of 4-APGA

*See fourth footnote on p. 24.

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has been separated from that of D·HCl inhibition in Fig. 3. An interesting hypothesis is that the dihydrotriazines may be selective inhibitors of the primary, DPN-linked reduction of PGA to dihydro-PGA in certain microbiological systems.

Early studies on the mechanism of sulphapyridine inhibition in bacterial systems suggested that it may be concerned with interference with DPN-mediated reactions.[47, 48] However, other studies[49, 50] with a number of sulphonamides, as well as the present observations in *Lactobacillus arabinosus*–PABA assay systems, do not support this hypothesis but rather indicate that the locus of sulphonamide inhibition differs from either that of 4-APGA or the D·HCl's.

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CHEMICAL AND BIOLOGICAL STUDIES ON 1:2-DIHYDRO-*s*-TRIAZINES—XIV

INHIBITION OF *DIPLOCOCCUS PNEUMONIAE*, TYPE II, *IN VITRO* and *IN VIVO* SYNERGISM WITH SULPHADIAZINE*

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Abstract—A number of 1:2-dihydro-*s*-triazines effectively inhibit *Diplococcus pneumoniae*, type II, *in vitro*. Differences in biological activity could be correlated with certain variations in structure and substitution in the molecule. Maximum activity was observed with 3′:4′-dichlorophenyl derivatives which have 2:2-dimethyl, 2-(*n*-decyl) or 2-(*n*-tridecyl) substitution in the triazine ring.

The dihydrotriazines exhibited synergism with both sulphadiazine and aminopterin. Synergism between sulphadiazine and aminopterin also was observed. Inhibition analyses indicate that these three classes of inhibitor differ with respect to loci of activity. The pattern of reversal of inhibition is consistent with previous evidence that the 1:2-dihydro-*s*-triazines interfere with a diphosphopyridine nucleotide-mediated reduction concerned with the metabolism of pteroylglutamic acid or its derivatives.

Several of these compounds are more effective than sulphadiazine in the therapy of experimental *Diplococcus pneumoniae*, Type II, infections in mice, and exhibit a profound synergism with sulphadiazine *in vivo*; combinations of concentrations of both inhibitors which are ineffective alone protect mice. Similar synergism also was observed *in vivo* with combinations of sulphadiazine and aminopterin, as well as with combinations of 1:2-dihydro-*s*-triazine and aminopterin.

INTRODUCTION

INHIBITION of the response of *Lactobacillus arabinosus* (17-5) no. 8014† to *p*-aminobenzoic acid (PABA) by a series of 1:2-dihydro-*s*-triazines (D·HCl's), and the synergistic activity of these compounds with sulphadiazine in this system[1] and in murine toxoplasmosis[2], suggested that the D·HCl's might exhibit a similar activity against infection induced in experimental animals by appropriate bacterial agents; for example, those which respond to sulphonamide therapy. The present report is concerned with the inhibition of *Diplococcus pneumoniae in vitro* and *in vivo* by certain dihydrotriazines.

EXPERIMENTAL

A mouse-virulent, S-phase strain of *Diplococcus pneumoniae*, type II,‡ maintained

*This investigation was supported in part by research grants C-1921 (C-3) and CY3335 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

†American type culture collection number.

‡Obtained through the courtesy of V. J. Fields, Department of Bacteriology, Harvard Medical School.

by mouse passage from 5% horse blood agar plates was used in these experiments. The methods of *in vitro* assay were those described for *Lactobacillus arabinosus*,[2] except that the medium was the tryptic digest broth described by Fields.[3] The basal medium contained, per millilitre, less than 0·001 μg of either pteroylglutamic acid (PGA) or citrovorum factor (CF), or both, as judged by microbiological assay with *Streptococcus faecalis*.

In the experiments, *in vivo*, mice of either sex, 20–25 g in weight, were infected intraperitoneally with 24 hr tryptic digest broth cultures of *D. pneumoniae* suitably diluted to contain in 0·1 ml the minimal dose which produced 100 per cent mortality in untreated mice within 72 hr. The D·HCl's, sodium sulphadiazine (sulpha) and aminopterin (4-aminopteroylglutamic acid) (4-APGA) were diluted appropriately with sterile glass-distilled water and the previously determined chronic maximum tolerated dose, and fractions thereof, were administered subcutaneously in approximately neutral solutions daily for 5 days, beginning on the day of infection. As recorded previously, the D·HCl's required careful administration to avoid untoward toxicity and cutaneous sloughing.[2] Each test group consisted of twenty or more treated and similar numbers of untreated mice. Mortality was observed daily for 10 days.

Structure–activity relationships

A comparison of the activity of representative dihydrotriazines is summarized in Table 1. Mono-halogen substitution in the phenyl ring had little effect, whereas

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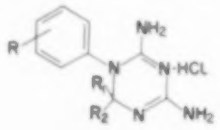
Table 1. Inhibitory activity of 1:2-dihydro-*s*-triazines *vs. D. pneumoniae*, type II, *in vitro*



Compound no.	Structure R	R_1	R_2	50% minimal inhibiting dose (μg/ml)
D-23·HCl	H	CH_3	CH_3	0·19
D-69·HCl	3′-Cl	CH_3	CH_3	0·175
D-20·HCl	4′-Cl	CH_3	CH_3	0·18
D-54·HCl	3′:4′-Cl_2	CH_3	CH_3	0·013
D-66·HCl	H	CH_3	C_2H_5	2·5
D-60·HCl	3′-Cl	H	C_2H_5	10+
D-43·HCl	4′-Cl	CH_3	C_2H_5	2·9
D-67·HCl	3′:4′-Cl_2	H	CH_3	1·25
D-75·HCl	3′:4′-Cl_2	CH_3	C_2H_5	0·31
D-68·HCl	2′-Cl	CH_3	CH_3	10+
D-98·HCl	2′-OCH_3	CH_3	CH_3	10+
D-40·HCl	3′-Cl	—$(CH_2)_5$—		10+
D-140·HCl	4′-SO_2NH_2	CH_3	CH_3	10+
Controls				
Na sulphadiazine (sulpha)				7·5
4-Aminopteroylglutamic acid (4-APGA)				3·0

di-halogen substitution resulted in a marked increase in activity. Those derivatives with asymmetrical substitution at the 2-position of the triazine ring were generally less active than the corresponding 2:2-dimethyl-substituted compounds. A compound incorporating the structural features of sulphanilamide into the D·HCl molecule (D-140·HCl)* was inactive, as it was in *Lactobacillus arabinosus*–PABA bioassay systems.[1]

Substitution at the *o*-position of the phenyl ring (D-68·HCl, D-98·HCl) or the presence of a large blocking group at the 2-position of the triazine ring (D-40·HCl) results in a marked decrease in activity (Table 1). Similarly, the anilinodihydrotriazines prepared by isomerization of the corresponding D·HCl derivatives[4, 5] also were inactive.

TABLE 2. EFFECT OF 2-(*n*-ALKYL) SUBSTITUTION AT 2-POSITION OF TRIAZINE RING ON ACTIVITY OF 1:2-DIHYDRO-*s*-TRIAZINES *vs. D. pneumoniae*, TYPE II, *in vitro*

Compound no.	Substitution*		50% minimal inhibiting dose (μg/ml)
	R_1	R_2	
R = H			
D-133·HCl	H	C_2H_5	10·0
D-113·HCl	H	n-C_6H_{13}	2·2
D-121·HCl	H	n-$C_{13}H_{27}$	0·028
R = 3′-Br			
D-130·HCl	H	C_2H_5	0·1
D-124·HCl	H	n-C_6H_{13}	0·28
D-122·HCl	H	n-$C_{11}H_{23}$	0·07
D-139·HCl	H	n-$C_{13}H_{27}$	0·028
R = 3′-Cl			
D-114·HCl	H	C_2H_5	2·8
D-110·HCl	H	n-C_6H_{13}	0·28
D-109·HCl	H	n-$C_{11}H_{23}$	0·028
R = 4′-Cl			
D-45·HCl	H	CH	3·1
D-84·HCl	H	n-C_6H_{13}	2·1
D-106·HCl	H	n-$C_{11}H_{23}$	0·028
R = 3′:4′-Cl_2			
D-67·HCl	H	CH_3	1·25
D-95·HCl	H	n-C_6H_{13}	1·7
D-116·HCl	H	n-$C_{10}H_{21}$	0·35
D-108·HCl	H	n-$C_{13}H_{27}$	0·021

*See Table 1 for structure.

The activity of the various phenyl-substituted derivatives is increased markedly by the presence of an *n*-hexyl substituent at the 2-position of the triazine ring, while *n*-undecyl to *n*-tridecyl substitution results in a further increase in activity (Table 2). These changes in biological activity consequent on alterations in structure are in general similar to those observed in other microbiological systems.[6]

Reversal of inhibition

The pattern of the non-competitive reversal of the D·HCl inhibition of *D. pneumoniae*

*The synthesis of this and the other previously undescribed dihydrotriazines considered here will be reported elsewhere.

is identical with that observed in the *Lactobacillus arabinosus*–PABA bioassay system,[1] except that natural CF,* thymine and thymidine are relatively ineffective. Similarly, the pattern of reversal of sulpha inhibition resembles that described for *Lactobacillus arabinosus*,[1] except that in the case of *D. pneumoniae*, natural CF, thymine and thymidine are ineffective, while excess guanine or xanthine is highly effective.

The inhibition of *D. pneumoniae* by 4-APGA, however, is distinctly different from that observed in *Lactobacillus arabinosus* [1] and other microbiological systems,[7, 8] in that reversal is not effected by PABA, PGA and its derivatives, or by DPN and its precursors, or by biological reducing agents.[1] So far, reversal of 4-APGA inhibition of *D. pneumoniae* has been effected only by relatively high concentrations of pteroic acid.[1*]

Synergism

Graphic analyses[9, 10] of the effects of combinations of sulpha and D·HCl or 4-APGA, and D·HCl and 4-APGA on the growth of *D. pneumoniae in vitro* indicate synergistic activity (Fig. 1), as was observed in *Lactobacillus arabinosus*–PABA bioassay systems.[1] Thus, these inhibitors involve different loci of activity with *D. pneumoniae*, as has been observed in other microbiological systems.[1, 7, 8]

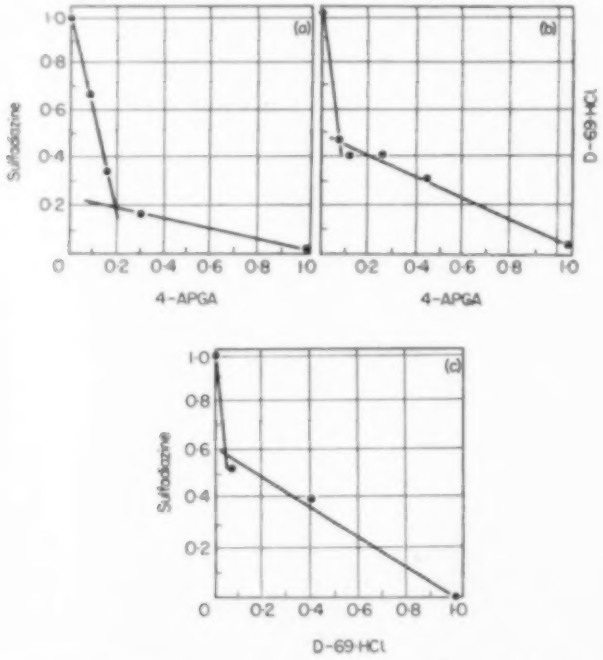


FIG. 1. Effect of combinations of inhibitors *vs. D. pneumoniae*, Type II, *in vitro*. (A) Sulphadiazine and 4-aminopteroylglutamic acid. (B) Sulphadiazine and 1:2-dihydro-*s*-triazine (D-69·HCl).* (C) 1:2-Dihydro-*s*-triazine (D-69·HCl)* and 4-aminopteroylglutamic acid.

Cf. Table 1 for structure.

*We wish to express our appreciation to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for generous samples of these compounds.

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Activity in vivo

The activity exhibited by representative D·HCl's *vs. D. pneumoniae*, type II, infection in mice is summarized in Table 3. Both the 2:2-dimethyl derivatives, D-20·HCl

TABLE 3. ACTIVITY OF 1:2-DIHYDRO-*s*-TRIAZINES *vs.* EXPERIMENTAL *D. pneumoniae*, TYPE II, INFECTIONS IN MICE

Compound no.	Substitution*			Dose (mg/kg)†	% Mortality/days		
	R	R_1	R_2		2	4	10
D-23·HCl	H	CH_3	CH_3	65	100		
D-69·HCl	3′-Cl	CH_3	CH_3	180	50	100	
				90	100	100	
D-20·HCl	4′-Cl‡	CH_3	CH_3	75	50	100	
				50	60	60	60
D-54·HCl	3′:4′-Cl_2	CH_3	CH_3	120	30	100	
				100	55	55	55
				25	45	90	90
D-133·HCl	H	H	C_2H_5	40	80	100	
D-114·HCl	3′-Cl	H	C_2H_5	100	50	100	
D-60·HCl	4′-Cl	H	C_2H_5	25	80	100	
D-63·HCl	3′:4′-Cl_2	H	C_2H_5	100	90	90	100
D-95·HCl	3′:4′-Cl_2	H	*n*-hexyl	100	100		
				50	100		
D-116·HCl	3′:4′-Cl_2	H	*n*-decyl	50	50	100	
				25	35	75	75
				12·5	65	100	
D-108·HCl	3′:4′-Cl_2	H	*n*-tridecyl	20	85	100	
				10	70	85	85
				5	90	100	
D-68·HCl	2′-Cl	CH_3	CH_3	100	100		
D-40·HCl	3′-Cl	——$(CH_2)_5$——		100	100		
D-140·HCl	4′-SO_2NH_2	CH_3	CH_3	100	100		
Controls	Untreated			—	80	100	
	Sulphadiazine			250	0	100	

*Cf. Table 1 for structure.
†Experiments with higher doses complicated by toxicity of D·HCl; lower doses less effective.
‡Corresponding anilinodihydrotriazine isomer ineffective at 100 mg/kg.

(4′-chlorophenyl) and D-54·HCl (3′:4′-dichlorophenyl), were more active than the corresponding derivatives which bear asymmetrical 2-carbon substituents at the 2-position of the triazine ring. The 3′-chlorophenyl-2:2-dimethyl derivative (D-69·HCl) exhibited some protective activity for 48 hr, but thereafter was ineffective. D-140·HCl, which incorporates the structural features of sulphanilamide into the D·HCl molecule, was ineffective, as were those derivatives bearing *o*-phenyl substituents (D-68·HCl) or a large-blocking group at the 2-position of the triazine ring (D-40·HCl) (Table 3).

The increasing activity of the phenyl-substituted derivatives with increasing length of the *n*-alkyl substituent at the 2-position of the triazine ring observed *in vitro* (Table 2)[1, 6] was not apparent in these experiments with *D. pneumoniae in vivo*. Derivatives bearing *n*-alkyl substituents in general were less active than the corresponding 2:2-dimethyl compounds, with the exception of the *n*-decyl (D-116·HCl) and the *n*-tridecyl (D-108·HCl) derivatives (Table 3). The presence of *n*-decyl or *n*-tridecyl substituents at the 2-position of the triazine ring also resulted in maximum activity against *D. pneumoniae in vivo* (Table 2).

The various D·HCl derivatives also exhibit synergistic activity with sulpha or 4-APGA *in vivo*. As illustrated in Table 4, combination therapy with concentrations of both inhibitors, either of which is completely ineffective alone, will protect mice against experimental *D. pneumoniae*, type II, infection. Combinations of D·HCl and 4-APGA also exhibit a similar synergistic activity (Table 4).

Table 4. Synergistic activity *vs.* experimental *D. pneumoniae*, type II, infections in mice

Therapy	% Mortality, 10th day	
	Expected	Observed
Sulpha, 100 mg/kg	100	100
D-54·HCl, 3·125 mg/kg*	100	100
Sulpha, 100 mg/kg + D-54·HCl, 3·125 mg/kg	100	20
Sulpha, 50 mg/kg + D-54·HCl, 3·125 mg/kg	100	0
Sulpha, 25 mg/kg + D-54·HCl, 3·125 mg/kg	100	60
Sulpha, 6·25 mg/kg	100	100
4-APGA, 0.3 mg/kg	100	100
Sulpha, 6·25 mg/kg + 4-APGA, 0·1 mg/kg	100	0
D-54·HCl, 6·25 mg/kg	100	80
D-54·HCl, 6·25 mg/kg + 4-APGA, 0·2 mg/kg	100	10
D-54·HCl, 3·125 mg/kg + 4-APGA, 0·3 mg/kg	100	40
Untreated controls	100	100

*Cf. Table 1 for structure.

DISCUSSION

It is clear that the locus of D·HCl inhibition of *D. pneumoniae*, type II, both *in vitro* and *in vivo*, differs from that involved with inhibition by sulpha or 4-APGA. However, the precise mechanism of action of the D·HCl's is not immediately apparent. The pattern of reversal of inhibition *in vitro* is consistent with previous evidence [1,8] that the D·HCl's interfere with a DPN-mediated reduction concerned with the metabolism of PGA or its derivatives. Experiments indicate that this strain of *D. pneumoniae* converts PGA and pteroic acid to one or more substances showing citrovorum factor activity as assayed by the usual method of bioassay using *Pediococcus cerevisiae* (formerly termed *Leuconostoc citrovorum*). This conversion appears to be unaffected by sulpha, but is inhibited by the D·HCl's and 4-APGA. Thus, the failure of natural CF to reverse D·HCl (and also 4-APGA or sulpha) inhibition suggests that either PABA or its derivatives, or both, may be converted to an active structure which differs from that of natural CF, such as those tetrahydro-PGA derivatives described in other biological systems,[11–14] or that *D. pneumoniae* is impermeable to natural CF. CF has been reported to be similarly ineffective in reversing the sulpha inhibition of *E. coli*.[15, 16]

Pteroic acid reverses both sulpha and 4-APGA inhibition, but is no more effective than PGA in reversing D·HCl inhibition, as would be expected from the results obtained with pteroic acid in *Lactobacillus arabinosus* systems.[1] The relative ineffectiveness of thymine and thymidine in reversing inhibition induced by any of the three inhibitors considered here suggests that impaired synthesis of thymine-containing

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compounds is not the growth-limiting factor under these experimental conditions. Further studies are now in progress.

Several of the D·HCl derivatives are more effective than sulpha in the therapy of *D. pneumoniae*, type II, infection in mice. Precise structure–activity studies *in vivo* are complicated by the inherent toxicity of many of the compounds in this series; however, it is apparent that the increasing activity *in vitro* consequent on increasing length of the *n*-alkyl substituent at the 2-position of the triazine ring (Table 2) does not obtain *in vivo* with the same strain of *D. pneumoniae*. Preliminary experiments indicate that this reduced activity *in vivo* may be due, at least in part, to the inactivating effect of serum on these *n*-alkyl derivatives.

The most interesting and probably the most significant aspect of these experiments *in vivo* is the profound synergism exhibited by combinations of D·HCl and sulpha. Synergistic activity with D·HCl and sulpha has been observed in experimental protozoan infections,[2,17] but such activity in experimental bacterial infections has not been reported previously in the literature. Biological activity resulting from combinations of small doses of inhibitors, any of which alone is devoid of activity even in much higher concentrations, as illustrated by the present studies, is unique. Studies on the usefulness of synergistic combinations of D·HCl and sulpha in other experimental systems are now in progress.

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METABOLISM *in vitro* OF THE SULPHHYDRYL AMINO ACIDS, L- AND D-PENICILLAMINE*

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Abstract—L-Penicillamine is not active as a substrate of L-amino acid oxidase. Its enantiomorph, however, is slowly oxidized by D-amino acid oxidase. Although neither isomer of penicillamine is desulphhydrated by L-cysteine desulphhydrase, the L-isomer inhibits the action of this enzyme. These findings are correlated with the enzyme-inhibiting and therapeutic activities of penicillamine.

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ALTHOUGH the inhibitory activity of L-penicillamine (the $\beta : \beta$-dimethyl analogue of cysteine) has been studied in various biological systems[1–8], and although the therapeutic effectiveness of the enantiomorphs of penicillamine in patients with Wilson's disease and with heavy metal intoxication has received recent attention,[9, 10, 11, 12] the activity of this amino acid as a substrate of the enzymes associated with cysteine catabolism has not been reported. In the experiments of the present communication, the activity of penicillamine as a substrate of L-amino acid oxidase, D-amino acid oxidase and L-cysteine desulphhydrase was investigated to obtain information regarding the metabolism of this amino acid.

EXPERIMENTAL

The oxidases of D-amino acid and L-amino acid were assayed by manometric methods described by Burton[14] and Ratner[14], respectively. *Agkistroden p. piscivorus* (cottonmouth moccasin) venom was used as the source of L-amino acid oxidase. The venom was obtained from Ross Allen Reptile Institute, Silver Springs, Florida. Flavin adenine dinucleotide (FAD) and crude hog kidney D-amino acid oxidase were purchased from the Sigma Chemical Company. Crude beef liver catalase was purchased from the Nutritional Biochemicals Corp.

Desulphhydrase activity was measured by the method described by Smythe.[14] The enzyme was prepared by making a saline extract of fresh rat liver. The saline extract was extracted with chloroform and the enzyme precipitated by adding acetone. A detailed procedure has been described[14]. The physical constants and sources of the penicillamine have been reported.[9, 10]

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RESULTS AND DISCUSSION

L-Penicillamine is not oxidatively deaminated by L-amino acid oxidase of moccasin venom (Fig. 1,A). The small amount of oxygen consumed (curve A) is the same as that noted when the enzyme is incubated without a substrate. Although D-penicillamine is metabolized by D-amino acid oxidase (Fig. 1,C) the rate of oxygen consumption is

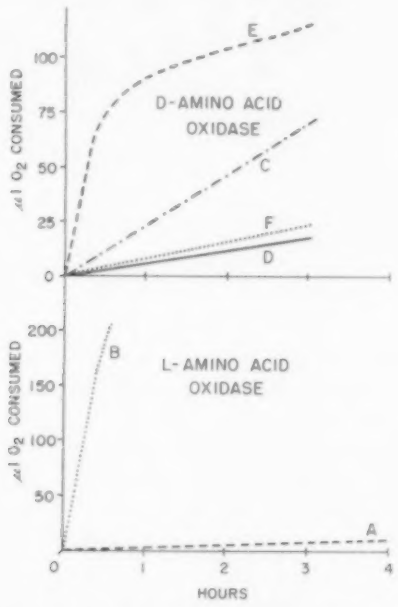

FIG. 1. Activity of penicillamine as a substrate for amino acid oxidase. *Curve A*: main compartment of Warburg vessel contained 5 mg *Agkistrodon p. piscivorus* venom dissolved in 0·5 ml 0·1 M KCl, 1·0 ml 0·2 M tris (hydroxymethyl) aminomethane buffer, pH 7·2; side bulb contained 100 µmoles L-penicillamine dissolved in 0·5 ml H_2O; 1·0 ml water was added; centre well contained 0·2 ml 20% NaOH. *Curve B*: conditions the same as above except 20 µmoles of L-cysteine substituted for L-penicillamine. The oxygen consumptions of the reaction mixtures without the venom have been substracted from the appropriate curves. *Curve C*: main compartment of vessel contained 8 mg crude hog kidney D-amino acid oxidase dissolved in 1 ml of buffer; 1·0 ml 0·1 M sodium pyrophosphate buffer, pH 8·3; 0·1 ml 10^{-4}M FAD, 40 µg crude beef liver catalase; side bulb contained 60 µmoles D-penicillamine dissolved in 0·2 ml water, pH 8·3; 0·2 ml water was added. Centre well conta ined 0·2 ml 20% NaOH. *Curve* D: same as those of curve C except D-amino acid oxidase omitted. *Curve E*: same as those of curve C except 10 µmoles D-cysteine substituted for D-penicillamine. *Curve F*: same as those of curve E except D-amino acid oxidase omitted. The gas phase in all experiments was air, the temperature 37 °C. Manometer readings made every 5 min.

low. The amount (8 mg) of the D-amino acid oxidase preparation used in the experiments described in Fig. 1 was twice that which is routinely used in this laboratory for D-amino acid oxidase studies. If 4 mg of the enzyme preparation are used, the rate of oxygen consumption is barely detectable when D-penicillamine is the substrate. Oxygen is consumed rapidly, however, if D-cysteine is the substrate in the presence of 4 mg of the enzyme preparation. This indicates that D-penicillamine is much less active than D-cysteine as a substrate of this enzyme.

When the action of a partially purified preparation of rat liver L-cysteine desulphhydrase on penicillamine is studied, neither enantiomorph is active as a substrate (Table 1). In fact, L-penicillamine inhibits the desulphhydration of L-cysteine (Table 1).

TABLE 1. ACTIVITY OF PENICILLAMINE AS A SUBSTRATE OF L-CYSTEINE DESULPHHYDRASE

Substrate (μmoles)	H_2S produced (μmoles)	Activity (%)
5·0 L-cysteine	3·01	100
5·0 L-penicillamine	0·0	0
5·0 D-penicillamine	0·0	0
5·0 L-cysteine + 5·0 L-penicillamine	0·53	18
5·0 L-cysteine + 5·0 D-penicillamine	2·07	69

Main compartment of Warburg vessel contained 33 mg partially purified rat liver cysteine desulphhydrase dissolved in 2·0 ml pH 7·4 phosphate buffer; side bulb contained 5·0 μmoles of each of the amino acids studied; total reaction volume 2·2 ml. Gas phase, nitrogen; incubated 2 hr at 37 °C. Centre well contained 0·1 ml of 1·0 M cadmium acetate.

Inhibition by the D-isomer is slight. Since pyridoxal-5-phosphate appears to be the coenzyme of cysteine desulphhydrase,[17, 18, 19] the inhibitory action of L-penicillamine is possibly the result of this amino acid's forming a thiazolidine carboxylic acid with the coenzyme. Such a reaction has been demonstrated between penicillamine and pyridoxal-5-phosphate in a non-enzymic preparation *in vitrio*, and has been suggested to occur with pyridoxal-5-phosphate enzymes by du Vigneaud and coworkers.[6, 7, 8]

There are many reactions by which cysteine is catabolized.[15] The ineffectiveness of cysteine in the treatment of heavy metal intoxication[9, 10] is probably the result of its rapid catabolism. The effectiveness of penicillamine[9, 10, 11, 12] as a metal-binding agent* appears to be due, in part, to its relative stability to metabolic degradation. This is substantiated by the present experiments with amino acid oxidases and cysteine desulphhydrase, and also by the observation that N-acetyl-DL-penicillamine is superior to DL-penicillamine in protecting rats against the lethal effects of mercuric chloride.[10] As would be expected, the N-acetyl derivative is not active as a substrate of either amino acid oxidase since the addition of the acetyl group has changed the amino group to an amide group which is not degraded by amino acid oxidase.[16]

It has been suggested that the growth inhibitory activity of L-penicillamine in the rat may be the result of the combination of this amino acid with pyridoxal-5-phosphate.[6, 7, 8] This anti-vitamin B_6 activity has been shown *in vitro* to depend on the presence of a free amino and a free sulphhydryl group.[7, 8] For this to be true *in vivo*, L-penicillamine should be inactive as a substrate of L-amino acid oxidase. This is substantiated by the present experiments.

Thus, oxidative deamination of D-penicillamine, as shown in the present experiments, and thiazolidine formation of L-penicillamine with pyridoxal-5-phosphate, as

*The combination of a metal ion with an electron donor results in a substance called a complex or co-ordination compound. If the substance which combines with the metal contains two or more donor groups so that one or more rings are formed the resulting structure is said to be a chelate.[13] If the metal combines with the electron donor and a ring structure is not formed, the resulting structure is called a complex. Although penicillamine has been shown to increase the excretion of many metals, it is not known whether it chelates or complexes.

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shown by du Vigneaud and coworkers,[7, 8] appear to be two of the routes for the metabolic inactivation of penicillamine. The inactivation of penicillamine by the amino acid oxidases, however, is considerably less than that of cysteine.

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METABOLIC STUDIES OF ^{32}P-LABELLED TRIETHYLENETHIOPHOSPHORAMIDE

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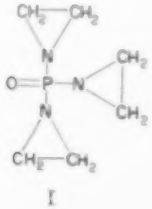
Abstract—Triethylenethiophosphoramide (ThioTEPA) labelled with radioactive phosphorus has been prepared and its metabolism examined in the rat, mouse, dog and rabbit. The drug was metabolized rapidly *in vivo*. The primary stage was the rapid replacement of S by O with the formation of TEPA. The mouse was exceptional in that the compound was degraded so that the principal radioactive metabolite excreted was inorganic phosphate In the rat, TEPA was the main metabolite; but the dog and rabbit excreted three other metabolites, although the major product was TEPA.

There was no specific localization of labelled material in the tissues of the mouse and rat apart from the retention of some radioactivity in the protein part of rat haemoglobin. The results suggest that this type of alkylating agent, in spite of its biological activity is not highly reactive chemically *in vivo*.

INTRODUCTION

IN AN earlier study of the distribution of ^{32}P-labelled triethylenephosphoramide (I) in the rat no specific localization of the drug was found in any of the tissues.[1] Over 80 per cent of the radioactive material appeared in the urine in 24 hr mainly as unchanged drug, suggesting that TEPA, a potent tumour inhibitor, was not very reactive chemically *in vivo*. These results contrasted with later findings by other workers which showed that labelled TEPA was virtually completely metabolized in the mouse to inorganic phosphate with no unchanged drug excreted.[2] In a recent paper on the distribution and fate of alkylating agents,[3] it was concluded that the excretion of a large proportion of unchanged TEPA in the rat "is incongruous in view of the postulated high degree of chemical and biological reactivity of this group of alkylating drugs".

In order to obtain more information on the fate of this type of compound we have examined the metabolism of ^{32}P-labelled triethylenethiophosphoramide (II) in the rat, mouse, dog and rabbit. Some preliminary results have been published elsewhere.[4]



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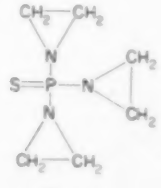

II

MATERIALS AND METHODS

Preparation of ^{32}P-*labelled triethylenethiophosphoramide*

Phosphorus trichloride, labelled with ^{32}P (35 mc), was converted to phosphorus sulphochloride.[5] To 2 g of the phosphorus trichloride, 0·04 g of aluminium trichloride and 0·465 g of sulphur were added. On heating, an exothermic reaction occurred and the product was distilled from the resultant clear brownish solution. The phosphorus sulphochloride was a colourless liquid, b.p. 122 °C (1·8 g). This product, dissolved in benzene (20 ml) was slowly added to a cooled mixture of triethylamine (3·22 g) and ethyleneimine (1·5 g) in dry benzene (20 ml), with stirring and cooling to 5 °C. After 30 min the mixture was filtered to remove precipitated triethylamine hydrochloride, the filtrate concentrated *in vacuo* and the solid residue recrystallized from benzene–petroleum ether (b.p. 40–60°). It formed colourless crystals (1·6 g), m.p. 50 °C and was chromatographically homogeneous in butanol: dioxan: 2 N ammonia ($R_f = 0·9$). Its specific activity was $1·76 \times 10^4$ counts/min per μg.

Distribution studies

The tissue distribution of radioactivity after a single injection of labelled thioTEPA (2·0 mg/kg, i.v.) was made in adult male rats of an American Wistar strain and in male mice. Freshly prepared aqueous solutions of the labelled compound were injected either intravenously or intraperitoneally. At selected times, groups of animals were exsanguinated by cardiac puncture under ether, the blood being collected in heparinized tubes and stored at 4 °C. Various tissue samples were removed and weighed. As a standard procedure solid tissues were digested for liquid counting, each weighed sample being heated with 2–3 ml of a solution of lithium hydroxide (10 per cent in 20 per cent aqueous ethanol) before diluting to 10 ml with water. The liquid counter tube had an efficiency of 9·1 percent for ^{32}P.

Metabolic studies

Dogs were anaesthetized with pentobarbitone (50 mg/kg), their ureters catheterized and urine samples collected as required after intravenous injection of the drug. Metabolic cages were used to collect urine from rabbits, rats and mice. In rats and mice killed soon after injection, however, urine was taken directly from the bladder.

Chromatography

Plasma and urine samples were chromatographed without pre-treatment. Single dimension ascending paper chromatography on Whatman no. 1 paper was used throughout. The main solvent system employed was freshly prepared butanol: dioxan: 2 N ammonia (4:1:5 v/v). The distribution of radioactivity on chromatograms was accurately determined with a multi-counter system[6] as well as by contact autoradiography using Ilford X-ray film.

RESULTS

Tissue distribution

Details of the distribution of radioactive material in the tissues of the rat and mouse are given in Table 1. In the mouse, a higher percentage of the dose was present in kidney tissue than in the rat—other mouse tissues contained less radioactivity, which corresponded with the more rapid clearance of the drug in this animal. The blood

level fell very rapidly, for 2 min after injection only 14 per cent of the administered radioactivity remained in the circulation of the rat and 10 per cent in the mouse. Similar low levels (less than 10 per cent of the radioactive dose) were found in the dog and rabbit a few minutes after injection. Blood from rats killed 9 days after injection contained a significant amount of radioactivity associated with the red cells. Examination of the haemoglobin from these cells, followed by separation of the haem from the globin, showed that all the ^{32}P was attached to the globin part of the molecule. A similar effect had been observed previously in rats given repeated injections of labelled TEPA.[1]

Table 1. Tissue distribution of radioactivity in the rat and mouse after intravenous injection of labelled thioTEPA (2 mg/kg). The 30 min, 200 min and 9 day values in both species are the means from five animals

Time after injection	Percentage of dose present at: 2 min	5 min	10 min	15 min	30 min	200 min	9 days
Rat							
Blood	14·2	11·7	10·7	9·8	8·3	4·5	0·53
Plasma	7·9	5·7	5·7	5·7	4·7	2·5	0·002
Spleen	0·36	0·51	0·47	0·62	0·44	0·26	0·09
Kidney	0·75	0·73	0·66	0·76	0·53	0·39	0·07
Liver	11·3	8·0	5·7	6·0	4·4	3·2	0·27
Testes	0·86	0·90	0·91	1·06	0·78	0·69	0·09
Mouse							
Blood	10·0	—	9·9	10·2	8·7	1·7	0·05
Plasma	4·7	5·6	5·5	5·6	5·1	1·0	0
Spleen	0·27	0·50	0·51	0·51	0·34	0·14	0·01
Kidney	2·0	2·8	2·6	2·7	3·1	1·2	0·06
Liver	6·8	8·9	7·8	8·7	7·8	3·9	0·20
Testes	0·41	0·44	0·62	0·60	0·43	0·17	0·02

Metabolism of thioTEPA

(1) *Rat*. Chromatography of urine collected ½, 2 and 24 hr after injection of thioTEPA showed that the radioactivity was almost entirely present as a single spot ($R_f = 0{\cdot}7$), corresponding exactly to labelled TEPA in the same solvent system. In the 24 hr sample two other labelled radioactive materials were present ($R_f = 0{\cdot}05$ and $R_f = 0{\cdot}15$) each representing about 5 per cent of the total radioactivity (Fig. 1). Chromatography of plasma 30 min after injection showed approximately equal proportions of unchanged drug and metabolite, $R_f = 0{\cdot}7$; 200 min after administration no unchanged material was present, the majority of radioactivity (75 per cent) being located in the TEPA zone ($R_f = 0{\cdot}7$) and the remainder about the origin (Fig. 1).

(2) *Mouse*. Urine samples collected ½ and 3 hr after injection of the drug were examined. The majority of radioactivity was present near the origin of chromatograms (Fig. 2), a position occupied by inorganic phosphate in the same solvent system. The remainder was spread out on both chromatograms from R_f 0·1 to 0·5 with a small peak at $R_f = 0{\cdot}4$–$0{\cdot}5$. Chromatography of plasma taken 30 and 200 min after injection revealed only a trace of unchanged drug; two components at $R_f = 0{\cdot}7$ (TEPA) and $R_f = 0{\cdot}05$ (phosphate) accounted for most of the radioactivity present (Fig. 2).

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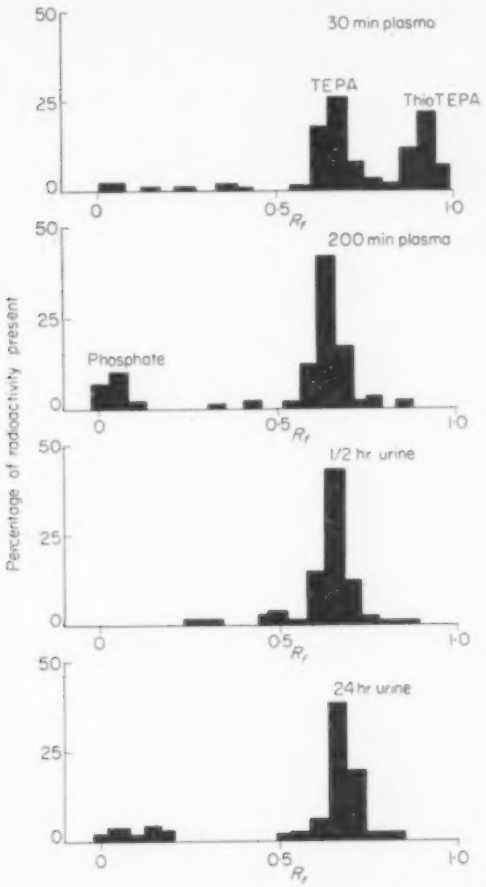


FIG. 1. Chromatograms showing the proportions of radioactive components present in plasma and urine from rats injected with radioactive thioTEPA (2 mg/kg, i.v.).

(3) *Dog*. A more detailed investigation was made of the radioactive materials present in plasma and urine shortly after injection of the labelled drug. Only urine samples collected less than 30 min after injection contained any unchanged drug, the main constituent being chromatographically identical with TEPA; three other components, were also present (R_f = 0·05, 0·2 and 0·5,) (Fig. 3). Because the blood radioactivity fell so rapidly chromatography of plasma had to be restricted to samples collected during the first $\frac{1}{2}$ hr after injection. The main component was TEPA although the metabolite, R_f = 0·5, found in urine, was also detected.

(4) *Rabbit*. Urine was collected for several days after injection, when 70 percent of the radioactive dose was accounted for in the combined samples (Table 2). The radioactive metabolites present were similar to those found in the dog (Fig. 3). In later samples the proportion of phosphate present increased but the major metabolite remained as TEPA (Table 2).

Table 2. The proportion of radioactive metabolites present in rabbit urine at different times after the injection of thioTEPA (2 mg/kg, i.v.). The amounts are expressed as percentages of the radioactive dose, the metabolites being separated by chromatography in butanol-dioxan 2 N ammonia (4:1:5 v/v).

Time (hr)	Radioactivity present (%age dose)	Composition of urine (as %age dose)			
		R_f 0·05	R_f 0·2	R_f 0·5	R_f 0·7
0–3	3·2	0·1	0·4	1·1	1·3
3–5	15·7	1·7	2·3	3·6	6·7
5–21	27·3	5·5	4·1	5·2	10·7
21–29	20·3	6·1	2·6	3·3	7·7
29–45	1·6				
45–74	1·2				
0–74	69·3	13·4	9·4	13·2	26·4

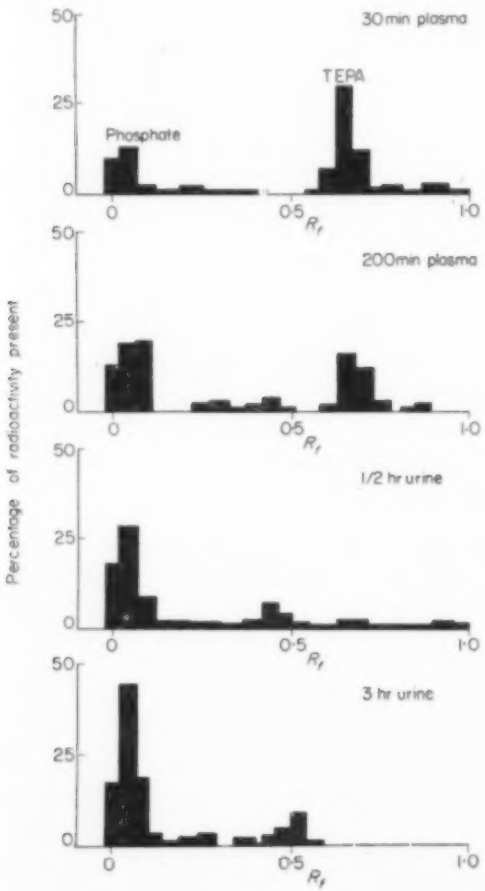


Fig. 2. Chromatograms showing the proportions of radioactive components present in plasma and urine from mice injected with radioactive thioTEPA (2 mg/kg, i.v.).

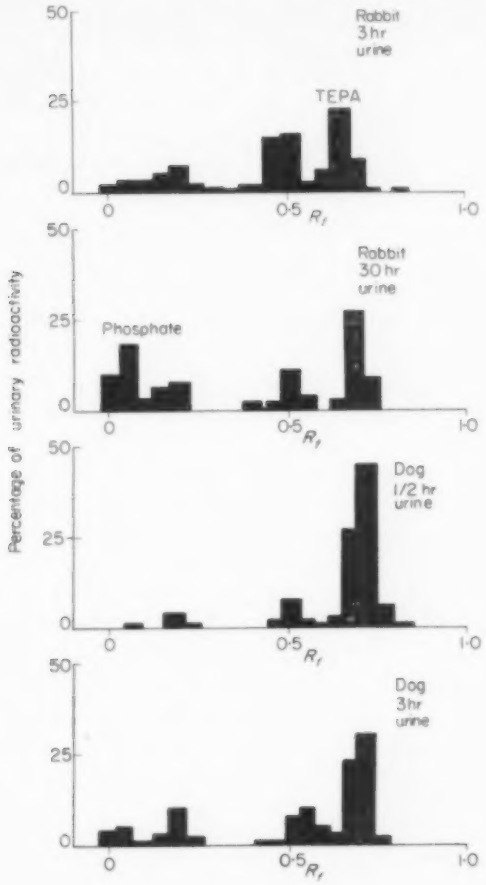


FIG. 3. Chromatograms showing the proportions of radioactive metabolites in the urine of dogs and rabbits injected with radioactive thioTEPA (2 mg/kg, i.v.).

Stability of thioTEPA. Samples of mouse and rat urine containing added thioTEPA, 8, 80 and 800 μg/ml of urine, were incubated at 37 °C. Chromatographic examination showed no deterioration of the drug after incubation for 2 hr; even after 20 hr only about 5 per cent had been hydrolysed to phosphate.

Excretion of ^{32}P-phosphate

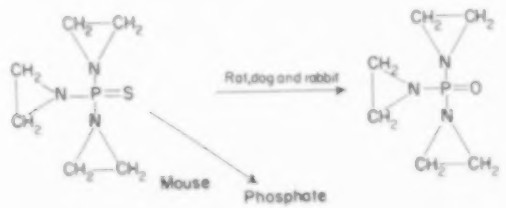
Five mice and five rats were injected with a solution of ^{32}P-labelled phosphate (1 mg/kg). Urine collected for 18 hr after injection contained about 14 per cent of the radioactive dose in each species.

DISCUSSION

The mechanism by which compounds containing ethyleneimine groups produce their biological effects is probably chemical reaction with cell components (alkylation). Other types of radiomimetic agents are known to react *in vivo* in this

way. For example, the aliphatic nitrogen mustard, methyl bis(2-chloroethyl)amine, labelled with ^{14}C, reacted rapidly in mice, 30–50 per cent being fixed in 6 hr.[7] The rate of excretion was correspondingly slow, the 24 hr urine containing less than 10 per cent of the radioactive dose. It may be presumed that most of the drug was bound in locations which are biologically unimportant. Attempts to study the reaction of alkylating agents with proteins and nucleic acids have been made *in vitro* but experimental conditions are very different from those *in vivo*.[8]

In our experiments using labelled thioTEPA, the rapid drop in circulating radioactivity after intravenous injection and the speed with which the tissues reached comparable specific activities indicate the ease with which the drug was distributed throughout the body. In the rat, 70 per cent of the radioactive dose was excreted in 18 hr, almost entirely as TEPA (Fig. 1). In the dog and rabbit the metabolic picture was more complicated, since four metabolites were separated; the major component, however, was TEPA (Fig. 3). A radical degradation of the drug occurred in the mouse with inorganic phosphate as the main metabolite although the plasma picture indicated that the first stage is the production of TEPA. In the urine there was no unchanged drug and only a trace of TEPA even $\frac{1}{2}$ hr after injection; later urine samples were similar in composition (Fig. 2). Compared with the other species examined the mouse is remarkable in its ability to metabolize the ethyleneimino-compound completely (Fig. 4). The phosphorus label provides no clue to the fate of the nitrogen rings, although earlier work in the mouse with ^{14}C ethyleneimino-labelled TEM suggests a general breakdown rather than a detoxication by alkylation of specific substances.[9] It is clear that the biologically active alkylating groups may remain largely unreacted in some species and so be excreted unchanged. In fact, the greater tolerance of the mouse for such compounds may be connected with ability to metabolize them rapidly.



In the mouse about 90 per cent of the radioactive dose of thioTEPA was excreted in 18 hr. Since only 14 per cent of a comparable dose of radioactive phosphate was recovered in the same period of time, a mechanism is indicated by means of which phosphate formed from the drug does not enter the general phosphate pool in this species. The inference is that the degradation of TEPA occurs in the mouse kidney—a point which is being further examined. Mouse urine, incidentally, does not possess the capacity to liberate phosphate from thioTEPA *in vitro*.

The only evidence of selective retention of labelled material was found in the red cells of rats (Table 1); its location was in the globin part of haemoglobin. This fixation of radioactivity in the protein part of haemoglobin had previously been noted after the injection of ^{32}P-labelled TEPA.[1] The drug appears to be directly involved in this

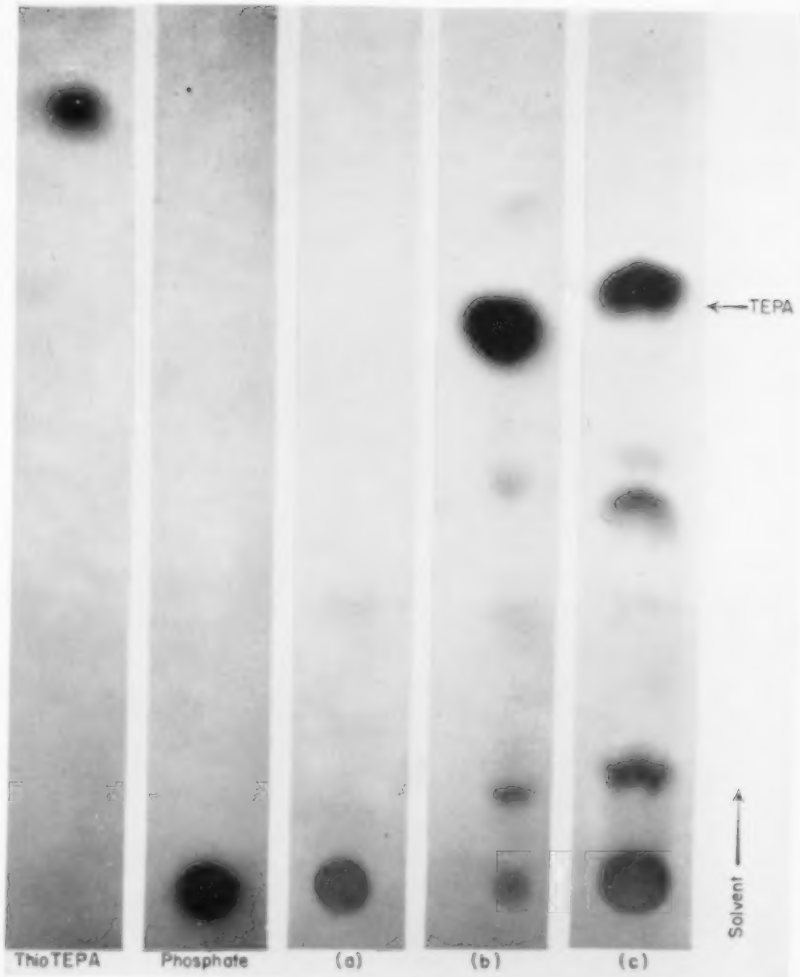


FIG. 4. Autoradiographs of chromatograms showing species differences in the radioactive metabolites present in urine collected 2 hr after giving ^{32}P-labelled thioTEPA (2 mg/kg, i.v.). Autoradiographs *A*, *B* and *C* are of mouse, rat and dog urine, respectively. For comparison the behaviour of ^{32}P-labelled thioTEPA and of phosphate are shown, using the same solvent system (butanol:dioxan:2 N ammonia).

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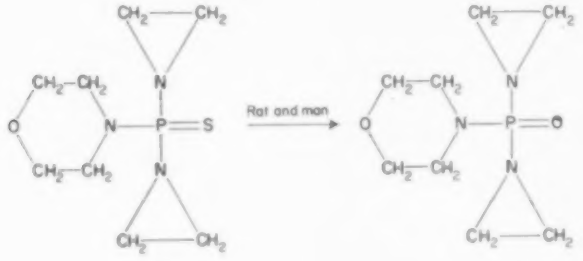
reaction with protein, for an equivalent dose of labelled phosphate did not result in the formation of labelled haemoglobin.

Species differences in the metabolism of thioTEPA reconcile the conflicting reports on the metabolism of TEPA. Our results with thioTEPA in mice agree with the known conversion of this substance to inorganic phosphate in this species.[2] A general assessment of the *in vivo* reactivity of ethyleneiminophosphoramides based on their fate in the mouse is therefore misleading. The metabolism of ethyleneimine compounds, such as thioTEPA, is more typically represented in other species where the excreted metabolites contain unreacted ethyleneimine groups. The metabolism in man and the rat of morpholinodiethylenethiophosphoramide (III), labelled with ^{32}P and ^{14}C, has been studied.[10] After injection of the drug into rats a considerable proportion of radioactive material in the urine (66 per cent of the 24 hr sample) was identified as morpholinodiethylenephosphoramide (IV), i.e. the sulphur had been replaced by oxygen. Using ^{14}C-morpholinodiethylenethiophosphoramide (III) the chief metabolite in man also was the desulphurized form (IV); only a trace of morpholine was present, and other ^{14}C compounds present were not identified.[10]



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Besides their antitumour activity, ethyleneimine compounds are known to produce marked effects on haematopoiesis[11] and spermatogenesis.[12] The evidence suggests that, although these compounds are widely distributed throughout the body, they are not highly reactive chemically and the marked biological effects are the result of the action of minute amounts of drug at specific locations. This type of compound, unlike more reactive kinds of alkylating agent, may ultimately yield information on the cell components involved in their biological effects provided a sufficiently detailed study of *in vivo* reaction sites can be made.

Acknowledgements—This work has been supported by grants made to this hospital by the British Empire Cancer Campaign.

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HIGHLY POTENT ANTIMETABOLITES OF SEROTONIN WITH LITTLE SEROTONIN-LIKE ACTION

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Abstract—Six new analogues of serotonin have been synthesized. All were made by condensation of N-phthalyl-1-benzyl-2-methyl-5-hydroxytryptamine with suitable alkyl halides to give alkyl indole ethers. The phthalyl group was removed by hydrazinolysis. Special condensing conditions were required for the alkylations because of the unreactivity of the phenol. Some of the new analogues were more potent as antimetabolites of serotonin than those previously known, when tested either on isolated rat uterus or *in vivo*. In addition, most of them had greatly reduced serotonin-like action on clam heart; in one this activity was undetectable. The most potent analogue, both as an anti-serotonin and as one without serotonin-like action in the heart-test, was 1-benzyl-2-methyl-5-tryptaminoxyacethydrazide. The importance of the lack of serotonin-like activity is pointed out.

STUDIES of the clinical effects of the antimetabolite of serotonin known as BAS, or 1-benzyl-2-methyl-5-methoxytryptamine, in the treatment of hypertension,[1–4] have raised two pharmaceutical chemical questions which the present work has sought to answer. These are: (a) can antimetabolites of serotonin be made which are even more potent than BAS? and, (b) can the gastric distress which is called forth in a small percentage of the patients by BAS be eliminated by modifying of the structure in such a way as to eliminate the serotonin-like action of BAS? Although BAS showed no serotonin-like effects on such test objects as the isolated rat uterus and in the blood pressure test with anaesthetized dogs,[2,5,6] it did exhibit such serotonin-like action to a slight extent in the blood pressure test with human beings[4] and in the clam heart assay. Because gastric contractions can be caused with serotonin, and because these might be associated with the gastric distress occasionally seen in a few patients taking BAS, it might be that this undesirable side-effect of the drug is the expression of its residual serotonin-like action on some tissues. Therefore, it seemed worth while to attempt to synthesize antimetabolites of serotonin which would lack detectable serotonin-like action on suitable test objects (e.g. clam hearts), and which would still possess the high anti-serotonin action of BAS in mammalian smooth muscles. These would also need to retain the other desirable features of BAS,[1] such as oral effectiveness, and especially the failure to induce psychotic changes.

Antimetabolites of serotonin lacking detectable serotonin-like action also might be useful for psychiatric experiments. As we have previously pointed out,[6–8] the fact that most existing analogues of serotonin which induce mental disturbances can be shown to be serotonin-like in tests such as that using clam hearts or human oligo-

*With the technical assistance of M. Gallagher and N. K. Campbell. Microanalyses were by Mr. T. Bella.

dendroglia[9] obscures the interpretation whether schizophrenia is to be viewed as a deficiency of cerebral serotonin or as an excess of it.[10] If an antimetabolite could be made which lacked serotonin-like action on any test object, but which still penetrated into the brain freely, some light might be thrown on this important question. In addition, if the disease should prove to be the result of excess cerebral serotonin, such an antimetabolite would have obvious therapeutic possibilities.

Several ideas for increasing the antiserotonin potency of BAS have been explored:

(a) Since it is well known that doubling the acetylcholine molecule, as in succinylcholine, or of its analogues, as in hexamethonium, leads to the formation of potent antagonists of this hormone, the doubling of the BAS molecule might similarly enhance potency. Compound (I) was therefore synthesized, but was found to be slightly less potent than BAS. Congeners of (I), in which the number of $-CH_2-$ groups bridging the indole rings was two and four instead of ten, were also made, but were not successfully purified. However, because the potency of the crude materials indicated that no great additional enhancement of potency was being realized, this approach was not pursued further.

FIG. 1. Structures of new antimetabolites of serotonin.

(b) Because Woolley[11] had shown that suitably substituted phenoxyethylamines were antimetabolites of serotonin, it was thought that if the phenoxy part were changed to oxytryptamine, anti-serotonin potency might be enhanced. Such an analogue would be twice an antiserotonin, once for the phenoxyethylamine moiety, and once for the alkyltryptamine moiety. Compound (II) was therefore prepared, and found to be very potent. Compound (III) was next prepared because it would be thrice an antiserotonin, once for the phenoxyethyl part, once for the alkyltryptamine part and once for the alkyl*iso*indolinyl part. Davis had found N-methylben*z*isoindoline to be antagonistic to serotonin.[12] Unfortunately, this substance (III) was less potent than

(II). However, it had the advantage of being able to penetrate into the central nervous system, as is shown by its effects on animals.

(c) When (II) and (III) were tested for serotonin-like action on clam hearts they were found to have much less of this sort of activity than had BAS. Therefore, it seemed possible that mere increase in size of the alkyl group which formed the ether part of BAS might be the way to decrease serotonin-like action. To test this idea, (IV), (V) and (VI) were synthesized and tested. The results indicated that more than size alone was involved. Nevertheless, one of these compounds, (IV), proved to have the properties desired (high anti-serotonin-activity and no serotonin-like activity).

The synthesis of these compounds was difficult. The amino group of BAS-phenol (1-benzyl-2-methyl-5-hydroxytryptamine) was protected by phthalylation,* and the phenolic hydroxyl was then reacted with a suitable alkyl halide, followed by removal of the phthalyl group by hydrazinolysis. The difficulty was that the phenolic group was so weakly acidic that it would scarcely react under the usual conditions with alkyl halides. The weakness of the phenol was readily shown by the fact that it (*viz.* 1-benzyl-2-methyl-3-phthalimidoethyl-5-hydroxyindole) could not be extracted from organic solvents by 0·1 N NaOH, and remained as the free phenol, not the sodium salt. With active halides, such as benzyl chloride or chloroacetamide, the desired products were obtained by long heating with the phenol in ethanolic sodium ethoxide, but with less reactive halides it was necessary to prepare the dry sodium salt of the phenol with sodium ethoxide, and to heat this dry salt for long periods with the halide.

Because of their high potency as anti-serotonins, and other desirable properties, some of these new compounds may possibly become useful both as laboratory tools and in the treatment of disease. The chemical names are too unwieldy for general use, and numbers lack distinguishability. Therefore, the trivial names shown in the figure were given to the most promising ones.

EXPERIMENTAL

N-*Phthalyl*-1-*benzyl*-2-*methyl*-5-*hydroxytryptamine*. Phthalic anhydride (4·5 g) and 1-benzyl.-2-methyl-5-hydroxytryptamine (7·7 g) (free gase)[5]† in 100 ml of toluene (dried over Na) were heated at 160–170°C in a open flask for 1 hr. The remaining toluene was removed under reduced pressure, and the residue was dissolved in 35 ml of ethanol plus 300 ml of ethyl acetate. This solution was washed once with 50 ml of cold aqueous 5 per cent sodium bicarbonate, then with water, dried with $MgSO_4$, and freed of solvent under reduced pressure. Whenever this or other phthalyl compounds were treated with aqueous alkali it was done rapidly in the cold. Even warm aqueous sodium bicarbonate (65° for 15 min) caused considerable opening of the phthalimide ring to give an acid (m.p. 90 °C) which was the *o*-carboxybenzoyl derivative of the tryptamine. The tarry residue from evaporation of the ethyl acetate was triturated very thoroughly for a long time with 600 ml of boiling benzene, and the soluble portion was concentrated under reduced pressure to 50 ml and stored overnight at 4 °C to give 8·0 g of crystals, m.p. 186–188 °C. Analyses are in Table 1.

*In preliminary work the amino group was protected by acetylation. However, when attempts were made to remove this group by acid hydrolysis of the final products extensive decomposition of the indole occurred even though the acid-labile positions (1) and (2) of the indole ring were protected.

†A quantity of this compound was generously supplied by Dr. K. Pfister of Merck, Sharp and Dohme.

TABLE 1. N-PHTHAPLYL-1-BENZYL-2-METHYL-TRYPTAMINES

Additional substituent	Recrystallizing solvent	M.P. (°C)	Empirical formula	Calculated C	H	N	Found C	H	N
5-Hydroxy	C_6H_6	186–188	$C_{26}H_{22}N_2O_2$	76·1	5·4	6·8	76·4	5·7	6·8
5-Oxyacetamide	$CHCl_3$-C_2H_5OH	218–220	$C_{28}H_{25}N_3O_4$	71·9	5·4	9·0	71·8	5·5	9·0
1′:10′-Decamethylenebis-(5-oxy)	C_2H_5OH	86–90	$C_{62}H_{62}N_4O_6$	77·7	6·5	5·8	77·0	6·8	6·0
5-Benzyloxy	C_2H_5OH	164–168	$C_{33}H_{28}N_2O_2$	79·2	5·6	5·6	79·0	5·8	5·8
5-(*p*-Methoxy-benzyloxy)	C_6H_6-hexane*	83-91†	$C_{34}H_{30}N_2O_4$	77·0	5·7	5·3	76·7	6·3	5·0
5-Phthalimidoethoxy	C_2H_5OH	—	$C_{30}H_{29}N_2O_5$	74·1	5·0	7·2	73·4	5·2	7·0

*Oily from ethanol; crystals could only be obtained by rapid addition of 5 vols. of hexane to a 10% solution in benzene.
†M.P. not sharpened by further purification.

N-*Phthalyl*-1-*benzyl*-2-*methyl*-5-*tryptaminoxy acetamide; general method for alkylation of the above phenol with active halides.* N-phthalyl-1-benzyl-2-methyl-5-hydroxytryptamine (10·5 g) was added to 100 ml of absolute ethanol in which had previously been dissolved 620 mg of sodium. Solution was effected by heating, and then 2·55 g of chloroacetamide was added. The mixture was refluxed anhydrously for 72 hr. Ethanol was removed under reduced pressure, the residue was washed with 100 ml of water and then heated with 500 ml of refluxing ethanol. The mixture was not filtered, but was cooled thoroughly and the crystals (6·4 g), m.p. 215–218°C., collected. An additional 150 mg was recovered from the mother liquor. Recrystallization from chloroform by addition of ethanol raised the m.p. to 218–220 °C. Analyses are given in Table 1.

Other N-*phthalyl*-1-*benzyl*-2-*methyltryptamines.* All other phthalyltryptamines, except the one described in the following section, were made by the general procedure described above. Because these other compounds were more soluble in ethanol, more concentrated solutions in this solvent were needed for crystallization. Gums which crystallized only with difficulty were frequently encountered. Details and analyses are summarized in Table 1. The alkyl halides used were: for the third compound, 1:10-decamethylene bromide; for the fourth, benzyl chloride; and for the fifth, *p*-methoxy-benzyl chloride.

N-*Phthalyl*-1-*benzyl*-2-*methyl*-5-(*β-phthalimidoethoxy*)-*tryptamine.* N-Phthalyl-1-benzyl-2-methyl-5-hydroxytryptamine (4·1 g) was dissolved in 100 ml of hot absolute ethanol in which had been previously dissolved 242 mg of sodium. Dry toluene (25 ml) was added and ethanol was removed by several evaporations under reduced pressure followed by additions of dry toluene, each time without complete removal of solvent. β-Bromoethylphthalimide (2·6 g) dissolved in 25 ml of dry toluene was added, and the resulting suspension was concentrated under reduced pressure to 10 ml. The flask was then heated under anhydrous conditions at 160–170 °C for 72 hr. The product was purified by several fractional crystallizations from ethanol. At first it came out of solution as a liquid, but it was finally obtained crystalline by cautious cooling of an ethanolic solution. Yield 3·44 g, m.p. indistinct, with extensive sintering beginning at 116 °C, and final melting at 126 °C. All attempts to prepare this substance by use of ethanol in the reaction mixture during the condensation (as in the preceding examples) failed. Analyses are given in Table 1.

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1-Benzyl-2-methyl-3-isoindolinylethyl-5-(β-aminoethoxy)-indole (*compound* (III)). A solution of 500 mg of lithium aluminium hydride in 100 ml of anhydrous ether was treated with 467 mg of N-phthalyl-1-benzyl-2-methyl-5-tryptaminoxy acetamide, and the mixture was stirred and refluxed under anhydrous conditions for 4 days. Excess hydride was decomposed by cautious addition of 6 ml of 50 % aqueous sodium potassium tartrate. The filtered ether phase was extracted with 55 ml of 0·1 N hydrochloric acid. The acid extract was immediately made alkaline with sodium hydroxide solution and the gummy precipitate which formed was extracted into ethyl acetate. The solvent was removed from the extract under reduced pressure and the residue was dissolved in ethanol and converted to the hydrochloride with two equivalents of ethanolic hydrogen chloride. The hydrochloride was crystallized by addition of ether; m.p. 158–162 °C. (Found: C, 66·8; H, 6·5; N, 8·2. Calc. for $C_{28}H_{33}Cl_2N_3O$; C, 67·4; H, 6·6; N, 8·4.)

1′:10′-Decamethylenebis-(1-benzyl-2-methyl-5-oxytryptamine) dihydrochloride (*compound* (I)). 1′:10′-Decamethylenebis-(*n*-phthalyl-1-benzyl-2-methyl-5-oxytryptamine) (86 mg) was dissolved in 30 ml ethanol plus 0·3 ml hydrazine hydrate and the solution was refluxed 7 hr. Most of the hydrazine was removed by repeated evaporation under reduced pressure, with additions of ethanol. The residue was suspended in 3 ml of 3 N ethanolic hydrogen chloride, heated to 50 °C for 5 min, cooled, and made alkaline with aqueous potassium hydroxide. The alkaline solution was heated for 15 min to open the phthalimide ring, thus rendering unextractable any unchanged starting material, and concentrated under reduced pressure to dryness. The residue was suspended in water, and the suspension was extracted twice with benzene. The benzene extract was evaporated and the residue was converted to the dihydrochloride by solution in ethanol, acidification, and evaporation. The dihydrochloride was then crystallized from hot water, in which it was only slightly soluble. Analyses and m.p. are in Table 2. The slight solubility of this compound in water severely limited its use in biological experiments.

TABLE 2. 1-BENZYL-2-METHYL TRYPTAMINES

Compound no.	Additional substituents	Recrystallizing solvent	M.P. (°C)	Empirical formula	Calculated C H N	Found C H N
(I)	1′:10′-Decamethylene bis-(5oxy) dihydrochloride	H_2O	146	$C_{46}H_{60}Cl_2N_4O_2$	– – 7·3	– – 7·5
(II)	5-(β-Aminoethoxy) dihydrochloride	C_2H_5OH	249*	$C_{20}H_{27}Cl_2N_3O$	60·6 6·8 10·6	61·7† 7·1 10·4
(IV)	5-Oxyacethydrazide	C_6H_6	131–134	$C_{20}H_{24}N_4O_2$	68·2 6·9 15·9	68·2 7·0 15·6
	5-Oxyacethydrazide monohydrochloride	C_2H_5OH‡	233–236	$C_{20}H_{25}ClN_4O_2$	61·7 6·4 14·4	61·9 6·8 14·3
(V)	5-Benzyloxyhydrochloride	H_2O	147–150	$C_{23}H_{27}ClN_2O$	– – 6·9	– – 6·9§
(VI)	5-(*p*-Methoxybenzyloxy)	C_6H_6-hex.	96–100	$C_{26}H_{28}N_2O_2$	78·0 7·1 7·0	77·6 7·1 6·8

*Began to soften at 238 °C.

†Compound was difficult to dry. Analyses after drying at 78 °C indicated 0·5 mole ethanol of crystallization. Analyses shown were of a sample dried 3 hr at 100 °C.

‡Can also be recrystallized from water.

§Dried at 60 °C; long drying at 100 °C caused some decomposition.

1-Benzyl-2-methyl-5-(β-aminoethoxy)-tryptamine dihydrochloride (*compound* (II)). Hydrazinolysis of N-phthalyl-1-benzyl-2-methyl-5-phthalimidoethoxytryptamine was carried out as in the preceding example, except that a greater excess of hydrazine (10 ml hydrazine hydrate plus 8 ml ethanol for 1·4 g) was required. The free base was a solid (m.p. 193 °C) which was extracted from the aqueous phase with chloroform more completely than with benzene. The dihydrochloride was soluble in water but was precipitated by excess chloride ions. This kind of behaviour has been found with several other tryptamines in this and preceding investigations.

1-Benzyl-2-methyl-5-tryptaminoxyacethydrazide (*compound* (IV)). When attempts were made to prepare the aminoethoxy analogue (compound (II)) by hydrazinolysis of N-phthalyl-1-benzyl-2-methyl-5-tryptaminoxyacetamide, followed by hydride reduction of the amide group, there was no success. The difficulty was that when the phthalyl compound was heated with 1 equivalent of hydrazine a mixture of starting material and the corresponding hydrazide was always obtained. It therefore proved impossible to prepare the unprotected tryptamine with the acetamide grouping on the ether oxygen by this route. For preparation of the hydrazide (compound (IV)) in acceptable yield, the following conditions were found suitable. N-Phthalyl-1-benzyl-2-methyl-5-tryptaminoxyacetamide (6·13 g) was dissolved by warming in 30 ml of hydrazine hydrate and 50 ml of absolute ethanol. The solution was heated on a steam bath for 1 hr and concentrated to dryness under reduced pressure. Ethanol was added and the evaporation repeated three times, in order to remove the excess hydrazine as completely as possible. Hydrolysis with hydrochloric acid and extraction of the free amine were carried out as described in the preceding examples, except that ethyl acetate rather than benzene was required for the extraction, and five extractions were necessary. Because of the basic nature of the hydrazide group it was necessary to adjust the pH to above 10 before making the ethyl acetate extractions. When this was not done, and only a slight excess of alkali was used as in the preceding methods, the yield was low, and the monohydrochloride of the desired product crystallized from the aqueous phase after long standing in the cold. The free base in the ethyl acetate extracts was freed of solvent and recrystallized by solution in ethanol, addition of benzene, and gradual replacement of the ethanol with benzene by frequent partial evaporations under reduced pressure. The yield was 2·7 g.

1-Benzyl-2-methyl-5-benzyloxytryptamine hydrochloride (*compound* (V)). This compound was made by hydrazinolysis of N-phthalyl-1-benzyl-2-methyl-5-benzyloxytryptamine according to the method described for compound (I) except that the refluxing was for 3 hr, and 1·2 equivalents of hydrazine were used. The free base could be crystallized from benzene, m.p. 116 °C after softening from about 110 °C.

1-Benzyl-2-methyl-5-(p-methoxybenzyloxy)-tryptamine (*compound* (VI)). Hydrazinolysis of the corresponding N-phthalyltryptamine with an excess of hydrazine as described for compounds (II) and (IV), yielded compound (VI). Chloroform rather than benzene was required to extract the free base from the alkaline aqueous solution. The hydrochloride was an oil which could not be crystallized, and which was extractable by water from chloroform only with difficulty. The free base was precipitated by addition of sodium hydroxide to an aqueous solution of the hydrochloride, and was crystallized from benzene by addition of hexane.

Antiserotonin activity in vitro *in rat uterus.* All compounds were compared in potency to BAS for their antiserotonin activity on the isolated rat uterus. In this assay the

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amount of analogue was determined which would prevent the contraction induced by an effective dose of serotonin as described earlier.[2,11] To increase the accuracy of comparison with BAS, one horn of the uterus of each rat was used to determine potency of BAS, and the other horn was used for assay of the new analogue. The same horn could not be used for both because all these new analogues, just like BAS,[2] were irreversible anti-serotonins. In all tests each dilution of the analogue was held in contact with the tissue for 10 min prior to addition of the challenging dose of serotonin. It is known that potency increases with increasing time of exposure to such analogues.[2,11] The specificity of most of the new anti-serotonins was demonstrated by showing that they did not antagonize the contractile action of acetylcholine. The results of the assays are summarized in Table 3, which contains the averages of at least three trials for each compound.

TABLE 3. SEROTONIN-LIKE AND ANTISEROTONIN ACTIVITIES OF SOME 1-BENZYL-2-METHYL TRYPTAMINES

Compound no.	Additional substituents	Antiserotonin activity		Serotonin-like‡ activity
		Rat uterus* (μg/ml.)	Mouse assay† (μg/mouse)	(%)
	5-Methoxy (or BAS)	0·2	250	3·0
	5-Hydroxy (or BAS-phenol)	—	50	0·3§
(I)	1′:10′-Decamethylenebis-(5-oxy)	0·5	—	
(II)	5-(β-Aminoethoxy)	0·1	100	0·1§
(III)	N-*Iso*Indoline-5-(β-aminoethoxy)	0·5	75	0·05**
(IV)	5-Oxyacethydrazide	0·05	10	less than 0·04**††
(V)	5-Benzyloxy	0·4	—	0·2-1·0‡‡
(VI)	5-(*p*-Methoxybenzyloxy)	0·02	5	1·0

*Expressed as the amount required to cause half-maximal inhibition of a contraction elicited by an amount of serotonin just sufficient to cause maximal contraction. All values were in direct comparison with the standard analogue (viz. BAS) as explained in the text.

†Amount required to protect half of the mice against diarrhoea induced by 1 mg 5-hydroxytryptophane per mouse.

‡Expressed as percentage of the activity of serotonin creatinine sulphate in causing increased amplitude of beat of clam hearts. Thus, 1 per cent means that 100 μg of analogue caused the same response as 1 μg of serotonin creatinine suphate.

§Dose–response curve less steep than for serotonin.

**The analogue decreased the base beat in two of five hearts (anti-activity?). In one of five no detectable serotonin-like or antiserotonin action.

††Detectable serotonin-like activity in only one of five hearts.

‡‡Considerable variation from heart to heart; almost inactive for some.

The potency of compounds (II), (IV) and (VI) was noteworthy. These are the most potent anti-serotonins known, as shown by the fact that the contraction of sensitive uteri (which will respond maximally to 0·01 μg of serotonin creatinine sulphate per ml) was reduced 50 per cent by 0·05 μg of compound (IV) or 0·02 μg of compound (VI) per ml. None of the new analogues showed any serotonin-like action on the rat uterus.

Anti-serotonin potency in vivo *in mice*. Some of the new analogues were compared in potency with BAS in the quantitative assay of Woolley,[13] in which the ability to prevent the contracting action of serotonin on intestines of mice is measured in normal animals. Results are summarized in Table 3. Again, the high potency of compounds

(IV) and (VI) is evident. Half of the animals were protected against the effects of 1 mg of DL-5-hydroxytryptophane by 10 μg. of compound (IV) per mouse or by 5 μg. of compound (VI).

Serotonin-like action in clam hearts. Most of the new compounds, as well as some previously known antimetabolites of serotonin, were assayed with isolated hearts of *Venus mercenaria*, for serotonin-like action.[14,15] Each was compared to serotonin for ability to increase the amplitude of beat as described earlier. Each dilution of analogue was held in contact with the tissue for 15 min in order to detect effects slow to appear.[11] The results are summarized in Table 3.

Although no detectable serotonin-like action of compound (IV) was observed, the results showed that in several hearts treated with this analogue there was some augmentation of the action of serotonin, when analogue and hormone were applied together. This may have indicated a trace of serotonin-like potency of this analogue.

The analogues were also tested on clam hearts for anti-serotonin activity. For this, graded doses of analogue were applied and the response to an effective challenging dose of serotonin was measured, as in the rat uterus assay. None of the compounds showed anti-serotonin activity. However, two of them (III and IV) reduced the amplitude of the beat of some of the hearts. This may have indicated some interference with the action of endogenous serotonin, because the decrease in amplitude could be overcome with serotonin.

DISCUSSION

The results of the present study show that it is possible to synthesize antimetabolites of serotonin with very high anti-serotonin potency for mammalian tissues, and ones which exhibit little, if any, serotonin-like potency on such invertebrate tissues as the clam heart. Because all previously tested analogues of serotonin which are antimetabolites in the usual tests with isolated smooth muscles have eventually been found to possess serotonin-like action on some sort of test object, the present results seem noteworthy. The usual test objects for serotonin-like activity have been mainly invertebrate muscles such as those of clam hearts or liver flukes, and might therefore seem of only academic interest, but when it was shown, as it has been for lysergic acid diethylamide, that such serotonin-like action also can be readily demonstrated on tissues of mammals, e.g. isolated brain cells of human beings,[9] or on those structures which control blood pressure in dogs,[6] one must pay closer attention to this phenomenon, especially since on this point depend some of the current arguments about the causation of schizophrenia,[10] and of hypertension.[1,4] It may be that in clam hearts and in the oligodendroglia of the brain the receptors for serotonin differ in structure from those in smooth muscles such as the rat uterus. This would allow many analogues of serotonin to combine with these heart or brain receptors and function in place of serotonin. In the receptors of the rat uterus, on the contrary, the analogues combine but are unable to carry on the functions of serotonin when they are so combined. Therefore they act as antagonists on these receptors. If this be true, an analogue such as compound (IV) of the present work must be pictured as being quite unable to combine with the receptors in clam hearts, even though it does unite with great avidity with those in rat uterus. The work now in progress,[16] the objective of which is to isolate and determine the chemical structures of the serotonin receptors, may eventually allow one to test these ideas directly. They are now only working hypotheses.

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The determination of serotonin-like action on clam hearts may be one useful way to assay an important property of antimetabolites of serotonin. Whether this is an adequate way, or whether an even more suitable test object can be found remains to be seen.*

The present work indicates that a very effective way to reduce, and even to eliminate, this serotonin-like property in 1-benzyl-2-methyl-5-hydroxytryptamines, is to attach certain alkyl groups as ethers to the oxygen at the 5-position. The indication was that to use a basic group such as an aminoethyl or acethydrazide radical was the most effective of those tried.

* The idea that the clam heart test may not be entirely adequate is shown by the following. BAS-phenol showed strong serotonin-like action on dog's blood pressure[5] but on clam heart was rather weak (Table 3).

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ENZYMIC HYDROLYSIS OF N:N-DIMETHYLCARBAMOYL FLUORIDE

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Abstract—The hydrolysis of N:N-dimethylcarbamoyl fluoride by rabbit tissues and by various vertebrate plasmata has been studied. The hydrolysis of this compound by rabbit liver and kidney homogenates involves an enzyme distinct from that hydrolysing di*iso*-propoxyphosphoryl fluoride. The same esterase in rabbit plasma probably hydrolyses dimethylcarbamoyl fluoride, di*iso*propoxyphosphoryl fluoride and *p*-nitrophenyl acetate. A similar result was obtained with human plasma. The major enzyme in rabbit plasma responsible for these reactions appears from electrophoretic studies to be the arylesterase (A-esterase). Aliesterase and cholinesterase of plasmata do not readily hydrolyse dimethylcarbamoyl fluoride. In addition to arylesterase, other factors of human serum may be involved in the hydrolysis reaction. The effect of certain metallic ions on the enzymic hydrolysis of dimethylcarbamoyl fluoride by rabbit plasma, liver and kidney is reported.

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MANY N-alkyl and N:N-dialkyl carbamates have been extensively studied with regard to their cholinesterase-inhibiting properties,[1,2,3] clinical usefulness,[1] and applicability as insecticides.[2,3] The N:N-dialkylcarbamoyl halides provide excellent model compounds for studying the mechanism of toxic action and detoxification of the N:N-dialkyl carbamates. The halides, described by Schrader[4] and studied by Myers,[5,6] are potent *in vivo* inhibitors of cholinesterases. The inhibitory reaction between this agent and acetylcholinesterase of rat brain is similar to that observed with organophosphorus esterase inhibitors.[7] Rats treated with dimethylcarbamoyl fluoride displayed a depression of brain and serum cholinesterase and aliesterase activity which was quite marked but of short duration, indicating a biological instability for this fluorocarbamate.[7] In the present investigation, the results obtained in studies on enzymes involved in the hydrolysis of dimethylcarbamoyl fluoride are reported.

MATERIALS AND METHODS

Compounds. N:N-Dimethylcarbamoyl fluoride (DCF), a colourless fluid of moderate water solubility, was provided by Dr. G. Schrader of Farbenfabriken-Bayer, Wuppertal-Elberfeld, Germany. Di*iso*propoxy phosphoryl fluoride (DFP) was provided by Dr. L.-E. Tammelin of the Research Institute of National Defence, Sweden.

Enzyme preparations. Tissues were obtained from a 2 kg male rabbit which was first bled as completely as possible by heart puncture. The washed erythrocytes and other organs were quickly frozen and kept at −10 °C until assayed. Tissue homogenates were prepared in bicarbonate-CO_2 buffer (pH 7·6) with a glass homogenizer. Plasmata

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from rabbit and other species were prepared from heparinized blood. The various fractions of human *post-partum* serum studied were those obtained by a partly modified method no. 6 of Cohn *et al.*[15]; these fractions were prepared and provided by AB KABI (Stockholm).

Esterase activity was determined by the bicarbonate method in the Warburg apparatus.[8] The compounds studied were dissolved in distilled water to which the enzyme and bicarbonate–CO_2 buffer (pH 7·6) was added to yield a total volume of 2·0 ml in the flask. All determinations were carried out at 37 °C, except as otherwise stated. Esterase activities were expressed in b_{30} values, i.e., μl CO_2 evolved per 30 min corrected for spontaneous hydrolysis of substrates and CO_2-evolution by tissues in the absence of added substrate.

Paper chromatography was carried out according to a described procedure.[9] The solvent was a *n*-butanol–ethanol–acetic acid–water mixture (8:2:1:3), and the chromatograms were developed by spraying with ninhydrin for the detection of methylamines.

Electrophoretic separation of plasma proteins was carried out according to a recently described method[10,11] on cellulose columns (3 cm × 40 cm) in veronal buffer (pH 8·4, $I = 0{\cdot}1$) at 5–11 °C, using 5 ml buffered plasma for each run and an applied current of 260 V and 60 mA. The duration of runs was 30 hr. The protein concentration of successive 3·0 ml fractions of the eluate was estimated by a modified Folin procedure,[12] and the enzyme activity of the fractions estimated manometrically.

RESULTS

Spontaneous hydrolysis. It was assumed that the spontaneous hydrolysis of DCF, as well as any enzymic hydrolysis yielded hydrofluoric acid, dimethylamine and carbon dioxide, by analogy with N:N-dimethylcarbamoyl chloride.[13,14] Under the conditions of the bicarbonate assay method only 1 mole of CO_2 would be expected from each mole of DCF hydrolysed. DFP yields under the same experimental conditions 2 molar equivalents of CO_2.

Neutral DCF solutions of concentrations below 0·01 M were stable for days when kept at a temperature below 20 °C. No degradation products from the solutions used could be detected by paper chromatography, and the inhibitory activity of solutions on cholinesterase was unaltered under the same conditions. Only a small amount of spontaneous hydrolysis could be detected with the manometric method at 37 °C and with high concentrations of DCF (0·1 M). When stronger alkaline solutions (0·01 M NaOH) of DCF were boiled for 4 hr, the formation of dimethylamine was ascertained by paper chromatography of the reaction mixture (Fig. 1).

Enzymic hydrolysis by various rabbit tissues. The activities of homogenates of certain rabbit tissues in the hydrolysis of DCF and DFP were compared (Fig. 2). With both substrates the plasma, liver and kidney were found to be the most active tissues. Of particular interest are the high relative activity of erythrocytes and the lowac tivity of the adrenal glands on DCF in comparison with DFP. From studies on swine and bovine tissues,[16] it was concluded that DFP and dimethylamido-ethoxy-phosphoryl cyanide (tabun) are split by the same enzyme of kidney and adrenal glands. This does not appear to be the case with the hydrolysis of DFP and DCF by rabbit kidney and adrenal glands.

FIG. 1. Paper chromatograms of DCF and products formed on incubation with arylesterase of human serum. Solvent: *n*-butanol-ethanol-acetic acid-water (8:2:1:3). Spots developed with ninhydrin. (1) $(CH_3)_2NH$, CH_3NH_2 and DCF, fresh aqueous solns; DCF alone gave no coloured spot. (2) DCF, 1% soln. in 0·01 M NaOH, boiled for 4 hr. (3) DCF, 10 mM, incubated with arylesterase for 24 hr at 22 °C in a bicarbonate–CO_2 buffer. No $(CH_3)_2NH$ was detected after incubation of DCF with cholinesterase and albumin, under the same experimental conditions.

Enzymic hydrolysis by various vertebrate plasmata. In a survey of various plasmata for activity in DCF hydrolysis, rabbit plasma was found to be the most active, with a b_{30} of 68/100 μl plasma. Relative to rabbit plasma, the cat and sheep were 23–25 per cent as active; the dog and cow, 12 per cent; and the horse, swine, guinea pig, mouse and chicken plasmata were only from 4 to 9 per cent as active in DCF hydrlysis. This order of activity of various plasmata correlated more closely with the hydrolysis of the phosphoryl cyanide (tabun),[16] than with that of 1-naphthyl-N-methylcarbamate[17] or with the activity of the arylesterases or cholinesterases of various plasmata.[10]

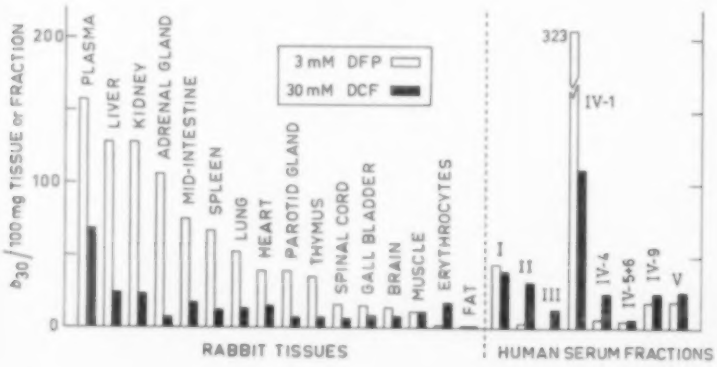

FIG. 2. Activity of rabbit tissue homogenates and human serum fractions in the hydrolysis of DCF and DFP. Determinations were made at 37 °C with 200 mg tissue or fraction for DCF, and 75 mg for DFP. Results are corrected for spontaneous hydrolysis of substrates and CO_2-evolution resulting with tissues or fractions in the absence of added substrate.

Enzymic hydrolysis by various fractions of human serum. Fractions from human *post-partum* serum were assayed for activity in the hydrolysis of DCF and DFP (Fig. 2). Although the highest activity against both substrates appeared in the fraction containing arylesterase (fraction IV-1), the activity in DCF hydrolysis was more generally distributed among the fractions than was the activity against DFP, tabun, tetraethyl pyrophosphate or diethyl *p*-nitrophenyl phosphate.[18]

Formation of dimethylamine during the enzymic hydrolysis. When DCF (10 mM) was incubated with a purified arylesterase preparation of human serum for 24 hr at 20 °C in a bicarbonate–CO_2 buffer, dimethylamine was detected after chromatography on paper of the reaction mixture (Fig. 1). No other nitrogen compounds were observed. Dimethylamine could not be detected when DCF was incubated under the same experimental conditions with purified preparations of cholinesterase or albumin of human serum.

Enzymic hydrolysis of mixtures of DCF and DFP. When mixed DCF and DFP of varying concentrations was incubated with rabbit plasma, the hydrolysis rates of the mixtures were found to be approximately equal to the theoretical values calculated on the assumption that the same enzyme acted on the two substrates (Table 1).

TABLE 1. HYDROLYSIS OF MIXTURES OF DCF AND DFP BY RABBIT PLASMA

(Determinations were made at 37 °C at 67 μl plasma per 2·00 ml reaction mixture).

mM DCF (*a*)	Rate of enzymic hydrolysis (b_{30})		
	DCF alone	3 mM DFP (*b*) + varying concs. of DCF	
		determined	calculated*
0	—	115	115
0·3	4	106	115
1	8	104	113
3	16	97	109
6	20	104	104
10	26	98	99
12	29	96	96·5
24	38	88	87·5
48	49	88	78·5

*On the assumption that DCF and DFP were hydrolysed by the same enzyme, applying the equation (for its derivation, see ref. 27), we get

$$\frac{V_a \frac{a}{K_a} + V_b \frac{b}{K_b}}{\frac{a}{K_a} + \frac{b}{K_b} + 1}$$

where V_a, V_b are the maximum velocities; a, b the concentrations; and K_a, K_b the Michaelis constants for DCF and DFP, respectively. V_a and K_a, deduced from cols. (1) and (2), are 60 and 12×10^{-3}. K_b for DFP and plasma *A*-esterase is 3×10^{-3}, which gives $V_b = 230$ when $a = 0$. For $b = 3 \times 10^{-3}$, the hydrolysis rate of the mixture is

$$\frac{a \times 60 + 230 \times 12 \times 10^{-3}}{a + 27 \times 10^{-3}}$$

However, at constant DFP concentration (3 mM) the values determined deviated from those calculated when the DCF concentration was higher than equimolar to

DFP. A possible explanation for this discrepancy would be that at higher DCF concentration a second enzyme (cf., Fig. 3) was coming in, and this hydrolysed DCF faster than DFP. In similar experiments with rabbit plasma pre-incubated with 0·1 mM DFP to inhibit all but the arylesterase activity, it was also found that the subsequent hydrolysis rates for DCF and *p*-nitrophenyl acetate in mixtures were approximately equal to those calculated, assuming that the two reactions were catalysed simultaneously by the same enzyme and provided the DCF-concentration was not higher than equimolar to the aromatic ester.

Electrophoretic separation of esterase active components in rabbit plasma. On electrophoretic separation of rabbit plasma on cellulose columns, it was found that probably two (A_1 and A_2) or more enzymes are involved in DCF hydrolysis and that these migrate coincidentally with a portion of the activity in the hydrolysis of DFP and *p*-nitrophenyl acetate (Fig. 3). The major enzyme involved seems to be identical with

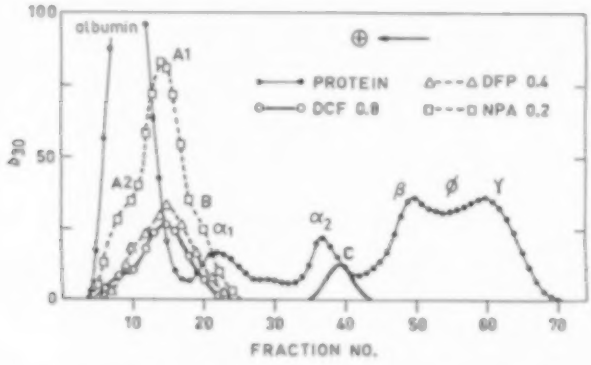

FIG. 3. Electrophoretic pattern of rabbit plasma showing elution position of fractions active in the hydrolysis of DCF, DFP and *p*-nitrophenyl acetate (NPA). b_{30}, esterase activity expressed in μl CO_2 evolved in 30 min with ml aliquot indicated within the graph. A_1, A_2, B and C represent elution positions for two arylesterases, aliesterase and cholinesterase, respectively. Protein curve is based on relative concentration values, and peaks are indicated with conventional symbols.

the arylesterase (A-esterase), which has been shown to hydrolyse dimethylamidoethoxyphosphoryl cyanide as well (AUGUSTINSSON, unpublished). The results indicate also that the DFP/DCF activity ratio differs for the two enzymes being higher for A_1 than A_2. Neither aliesterase nor cholinesterase hydrolysed DCF or DFP.

Effect of Mn^{2+} *and* Co^{2+}. The effect of Mn^{2+} and CO^{2+} on the hydrolysis of DFP and DCF by rabbit plasma, kidney and liver was investigated (Table 2). Mn^{2+} and Co^{2+} activated the DFP-hydrolysing enzymes of liver and kidney as was previously noted with many species,[19] but resulted in inhibition of the DFP-hydrolysing esterases of rabbit plasma as has also been previously noted by Mounter.[20] The tabun-hydrolysis was similarly influenced by those ions.[21] With DCF as substrate, Mn^{2+} failed to activate, and Co^{2+} was inhibitory to, DCF hydrolysis by liver and kidney. However, with plasma the degree of inhibition by Mn^{2+} and Co^{2+} was similar for both substrates. These results indicate that different enzymes in liver and kidney are primarily responsible for DCF and DFP hydrolysis, whereas the same plasma enzyme(s) may contribute to the hydrolysis of both substrates.

TABLE 2. EFFECT OF Mn^{2+} AND Co^{2+} ON THE HYDROLYSIS OF DCF AND DFP BY RABBIT PLASMA, LIVER AND KIDNEY

Whole plasma or 20 per cent tissue homogenates were dialysed against distilled water before assay. Buffer for assay was 0·04 M $NaHCO_3$–CO_2 at pH 7·6. $MnSO_4$ or $CoCl_2$ was incubated with the enzyme for 60 min at 37 °C before adding the substrate. Results are corrected for spontaneous hydrolysis of substrates in the presence and absence of added cations and for CO_2 evolution from tissues in the absence of added substrate. Enzyme activity (b_{30}) refers to 100 μl plasma or 100 mg tissue. Ratios refer to activity with 1·0 mM metal ion divided by activity with no added metal ion.

Tissue	DCF (30 mM)			DFP (3 mM)		
	b_{30}	Ratios		b_{30}	Ratios	
		Mn^{2+}	Co^{2+}		Mn^{2+}	Co^{2+}
Plasma	75	0·79	0·02	120	0·79	0·02
Liver	15	0·92	0·59	67	1·64	1·68
Kidney	15	0·99	0·64	79	2·00	1·75

Effect of various metallic ions on the enzymic hydrolysis by rabbit plasma. A more complete study was made of the rabbit plasma systems hydrolysing DCF and DFP, since rabbit plasma was most active in DCF hydrolysis of those examined, and Mounter[19] has concluded that the A-esterase (arylesterase) of rabbit plasma is responsible for DFP hydrolysis. The relative effects were determined of several possible

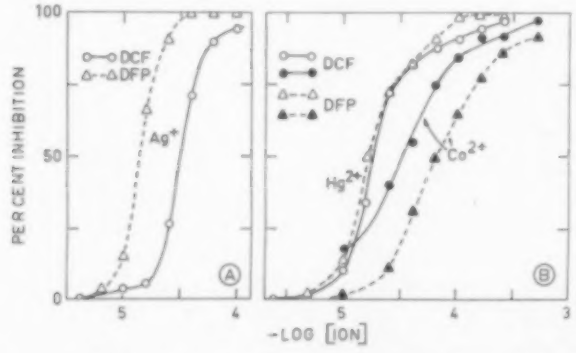

FIG. 4. Inhibitory activity of certain metallic ions on the enzymic hydrolysis of DCF and DFP by rabbit plasma. The plasma was dialysed against distilled water and used at 100 μl per 2·00 ml reaction mixture for assay against 30 mM DCF and at 50 or 100 μl against 3 mM DFP. The buffer solution used was 0·04 M $NaHCO_3$–CO_2 at pH 7·6. The metallic salts ($AgNO_3$, $HgCl_2$ and $CoCl_2$) were incubated with the enzyme for 60 min at 37 °C before adding the substrate. Results are corrected for spontaneous substrate hydrolysis. A: enzyme concentration for DCF twice that for DFP. B: the same enzyme concentration for DCF and DFP. Values for pI_{50} are as follows:

Substrate	Ag^+	Hg^{2+}	Co^{2+}
DCF	4·5	4·75	4·2
DFP	4·85	4·8	4·5

cationic inhibitors of the enzymes hydrolysing DCF and DFP. For DCF determinations the enzyme concentration was usually twice and the substrate concentration ten times that for DFP. At 1·0 mM, Ca^{2+} and Mg^{2+}, gave slight inhibitory effects, Mn^{2+}, Ba^{2+} and Sr^{2+} were more active inhibitors, and Co^{2+}, Cu^{2+}, Ag^{+} and Hg^{2+} gave almost complete inhibition. Similar results were obtained with both substrates. On further dilution of the more active inhibitors, it was found that the effect on the enzymes hydrolysing both substrates increased in the order: $Cu^{2+} < Co^{2+} < Hg^{2+}, Ag^{+}$. Inhibition curves for the three most active cations are shown in Fig. 4. Where the same enzyme concentration was used for assay against DCF and DFP, the inhibition curves with Hg^{2+} were almost coincidental, but when twice the enzyme concentration was used for DCF as for DFP it required twice as much Ag^{+} to effect a similar degree of inhibition of DCF hydrolysis. However, where the same enzyme concentration was used to assay against the two substrates, different inhibition curves resulted with Co^{2+}. This effect was not further investigated nor was the high inhibitory effect of Co^{2+} found in these experiments compared with a much lower Co^{2+} inhibition of DFP hydrolysis by rabbit serum reported previously.[20] The differences in enzyme or substrate levels, or the nature of the substrate, may be contributing factors. It has been shown recently[21] that Sr^{2+} will activate the human serum enzyme hydrolysing tabun while this cation inhibits the reaction on DFP by what appears to be the same enzyme. However, as the experiments in Fig. 4 were carried out with a mixture of the two enzymes A_1 and A_2 (Fig. 3), the discrepancies noted would be explained if A_2 were less sensitive to Co^{2+}, but more sensitive to Ag^{+}, than A_1, and if A_1 and A_2 were equally sensitive to Mg^{2+}.

CONCLUSIONS

Several different enzyme systems appear to be involved in the detoxification of N-alkyl and N:N-dialkylcarbamates. Although cholinesterases will apparently hydrolyse certain carbamates,[7,22–27] the turn overnumber is so low that these enzymes might not be considered of importance as a major detoxification mechanism. Plasma albumin has been implicated as a factor of possible importance in the detoxification of 1-naphthyl-N-methylcarbamate[17] and *p*-nitrophenyl-N-alkylcarbamates.[24] However, neither plasma albumin nor plasma arylesterase readily hydrolyse the more stable N:N-dialkylcarbamates.

As shown in the present investigation, one of the least stable of these N:N-dialkyl carbamates, dimethylcarbamoyl fluoride, is hydrolysed by enzyme(s) in human and rabbit plasmata which may also be involved in the hydrolysis of di*iso*propoxy-phosphoryl fluoride and *p*-nitrophenyl acetate. One of these enzymes is probably identical with arylesterase (A-esterase). Aldridge[25] and Mounter[20] have previously concluded that this esterase of rabbit plasma also hydrolyses tetraethylpyrophosphate and diethyl-*p*-nitrophenyl phosphate. The hydrolysis of DCF by human plasma is probably determined by more factors than the hydrolysis of organophosphorus compounds.[26] In rabbit liver and kidney, different enzymes are primarily involved in the hydrolysis of dimethylcarbamoyl fluoride and di*iso*propoxy-phosphoryl fluoride.

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provided by Dr. G. Schrader of Farbenfabriken-Bayer, Wuppertal-Elberfeld, Germany, di*iso*propoxyphosphoryl fluoride by Dr. L.-E. Tammelin of the Research Institute of National Defence, Sweden, and rabbit plasma and tissues by Dr. G. von Ehrenstein of the Institute of Organic Chemistry and Biochemistry, University of Stockholm. Fractions of human serum were made available for this study by AB KABI, Stockholm. We are grateful to Mrs Gunnel Jonsson for her skilful technical assistance.

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BIOCHEMICAL AND PHARMACOLOGICAL STUDIES ON N-METHYLATED CARBAMOYLCHOLINES

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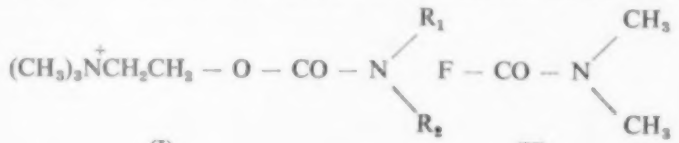
Abstract—N-methyl- and N:N-dimethyl-carbamoylcholines are highly stable compounds in aqueous solution and can only be hydrolysed to yield choline and methylamines when boiled for several hours in alkaline solutions. These esters are not degradated by butyrylcholinesterase, acetylarylesterase and albumin of human serum, or by acetylcholinesterase of electric organ. Cholinesterases are inhibited by concentrations less than 10^{-4} M. The inhibition is progressive and at least partly irreversible, probably owing to carbamoylation of the esterastic site of the enzyme molecule. In comparison with carbamoylcholine the two substituted derivatives are less toxic and have in general weaker pharmacological activities. However, they have more pronounced ganglion stimulating effects and are respiratory stimulants. Potentiation of the response to acetylcholine could not be demonstrated. On the frog rectus both derivatives are approximately four times more potent than carbamoylcholine and five times less potent than acetylcholine. Carbamoylcholine is as potent as acetylcholine on the guinea-pig ileum, while the monomethyl derivative is 40 times, and the dimethyl derivative 150 times less, potent than carbamoylcholine.

CARBAMOYLCHOLINE (I, $R_1 = R_2 = H$) has been extensively studied biochemically and pharmacologically,[1,2] but its carbamoyl N-substituted derivatives have been little investigated[3,4] with the exception of the closely related dibutylcarbamate of ethyl (2-hydroxylethyl) dimethylammonium (dibutoline).[4] The synthesis of N-methylated carbamoylcholines was reported previously[5,6] and their miotic action noticed.[4] It is known that substitution in the aminogroup of carbamoylcholine very greatly reduces the muscarine-like activities and even produces molecules which are antagonistic to acetylcholine, i.e. atropine-like.[4]

In connexion with biochemical studies of a series of N-alkyl- and N:N-diakyl carbamates,[7] it was demonstrated that arylesterases and plasma albumin[8,9] are active in the decomposition of these compounds. Owing to the marked influence of N-alkyl substitution in carbamates on both the biochemical and pharmacological properties and to lack of knowledge of these properties of N-methyl-(MeCbCh) (I, $R_1 = Me$, $R_2 = H$) and N:N-dimethylcarbamoylcholine (Me_2CbCh) (I, $R_1 = R_2 = Me$), these compounds were studied in the present investigation. Some results with N:N-dimethylcarbamoylfluoride (DCF) (II) are inserted for comparison.



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MATERIAL AND METHODS

Compounds tested

The two N-methylated carbamoylcholine chlorides were synthesized and provided by Dr. H. Gysin and Dr. A. Margot of J. R. Geigy, Basle, Switzerland. The melting points for the two compounds, determined with a Kofler block on minute crystals, were: MeCbCh, 173–4 °C; Me_2CbCh, 181–4 °C; Sprinson[6] obtained 178–80 °C and 185–7 °C, respectively. Both compounds were highly hygroscopic. Carbamoylcholine chloride was a commercial product (Doryl). DCF was provided by Dr. G. Schrader of Farbenfabriken-Bayer, Wuppertal-Elberfeld, Germany.

Enzyme preparations

Cholinesterase, arylesterase and albumin were purified preparations from human serum.[10] A purified preparation of acetylcholinesterase from *Torpedo* electric organ was also used.

Biochemical methods

Paper chromatography was carried out according to a method recently described.[11] The solvent used was a *n*-butanol–ethanol–acetic acid–water mixture (8:2:1:3). The ascending technique was used and chromatograms were developed by spraying with ninhydrin for the detection of dimethyl- and monomethylamine, and with dipicrylamine (0·2 g in 50 ml acetone and 50 ml distilled water) for the detection of choline and its derivatives.

Esterase activity was determined with the Warburg technique[12] using acetylcholine chloride as substrate for cholinesterases, and expressed in b_{30} values, i.e. μl CO_2 evolved in 30 min from a bicarbonate–CO_2 buffer with corrections for nonenzymic hydrolysis of the substrate.

Pharmacological methods

Acute toxicity. The acute toxicity of the compounds was tested by intraperitoneal injection in albino mice weighing about 20 g. The compounds were dissolved in isotonic saline, and the injected volume did not exceed 0·4 ml. The determinations were designed as rough screenings in groups of ten mice.

Experiments in the anaesthetized cat. The effects of the esters were studied in eight cats, anaesthetized with sodium pentobarbital (30–40 mg/kg) administered intraperitoneally. Drugs were injected intravenously through a plastic cannula in a femoral vein. The following parameters were recorded by passing the output of suitably arranged transducers into a Grass Model 5 Polygraph.

Respiration was recorded as pressure differences in the tracheal cannula by means of a pressure transducer (Model PT5, Grass Instrument Corporation). Blood pressure was recorded by means of a Statham Electromanometer from the left carotid artery. Pulse rate was estimated by the use of an interval recorder as described by Goldschmith and Lindgren.[13] The effects on neuromuscular transmission were demonstrated by recording semi-isometric contractions of the gastronemius muscle in response to supramaximal electrical stimulation of the sciatic nerve by means of a Grass stimulator, Model S4. The stimuli were applied to the nerve distally to a crushed region by means of shielded silver electrodes. A force-displacement transducer Model FT10, Grass Instrument Corporation was used for recording the muscle contractions.

Contractions of the nictitating membrane were recorded by means of a force-displacement transducer, FT03. The excitability of the membrane was tested by preganglionic stimulation of the exposed cervical sympathetic trunk for 10 sec with rectangular pulses at a frequency of 30 c/s. Duodenal intraluminal pressure was recorded by means of a Statham electromanometer connected to a balloon, inserted through the oesophagus into the duodenum. The insertion necessitated laparotomy. The balloon and the cannula were filled with water. Femoral arterial blood flow was recorded by photoelectric drop recording with the aid of an interval recorder.[14] The equipment allowed the simultaneous recording of five parameters.

Frog rectus abdominis muscle preparation. The rectus muscle was suspended in a 3 ml bath containing eserinized frog-Ringer solution at room temperature. The preparation was aerated and left in contact with the drugs for exactly 90 sec. The response was compared with that of an acetylcholine standard and expressed as the relative molar potency in percentage units (i.e. as the number of moles of acetylcholine giving a response equivalent to that of 100 moles of the test substance).

Guinea-pig ileum preparation. A test bath of 10 ml was used. An automatic timer regulated the cycle of operations except for the addition of the compounds, which was done manually in a small volume on top of the test bath. The results were expressed as the relative molar potency as defined above.

RESULTS

Stability tests

Aqueous solutions of both MeCbCh and Me_2CbCh were very stable and could be kept at room temperature (22 °C) without any loss in biochemical and pharmacological activities. Even in 0·01 M sodium hydroxide these compounds were stable for several days. Proof of this stability was obtained by paper chromatography (Fig. 1) and the

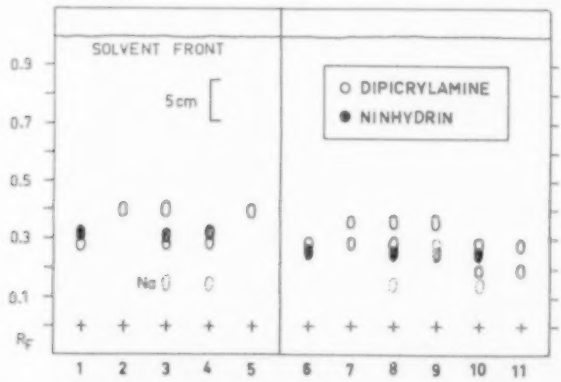

FIG. 1. Paper chromatograms of neutral and alkaline solutions (1 per cent) of MeCbCh and Me_2CbCh. Solvent, *n*-butanol–ethanol–acetic acid–water (8:2:1:3). Alkaline solutions made with 0·01 M NaOH. (1) Choline and Me_2NH; (2) Me_2CbCh, neutral soln.; (3) Me_2CbCh, alkaline soln. boiled for 4 hr; (4) choline and Me_2NH, alkaline soln. boiled for 4 hr; (5)Me_2CbCh, neutral soln. boiled for 4 hr; (6) choline and $MeNH_2$; (7) MeCbCh and choline; (8) MeCbCh, alkaline soln. boiled for 4 hr; (9) MeCbCh, neutral soln. boiled for 4 hr; (10) carbamoylcholine and $MeNH_2$, alkaline soln. boiled for 4 hr; (11) carbamoylcholine and choline.

TABLE 1. STABILITY OF AQUEOUS SOLUTIONS OF MeCbCh AND Me_2CbCh MEASURED BY THEIR CHOLINESTERASE INHIBITING ACTIVITIES

(50 mM solutions of the choline esters were kept at 22 °C for various periods of time and tested against human serum cholinesterase with acetylcholine (10 mM) as substrate. Inhibiting activity expressed as the percentage inhibition obtained with dilutions of the stock solutions to give 0·01 mM choline ester in the reaction mixture. Values in brackets refer to 1·0 mM stock solutions in 0·01 M NaOH.)

Inhibitor	Zero time	24 hr	12 days
MeCbCh	63 (62)	64 (68)	65
Me_2CbCh	34	38	31

esterase inhibition technique (Table 1). No hydrolysis products were observed on paper chromatograms, and the esterase inhibition was unaltered with neutral and alkaline solutions, kept for various periods of time. Neutral solutions of the compounds kept for weeks did not alter their pharmacological activities. When the alkaline solutions were boiled for 4 hr the compounds were split into choline and methylamines, detected on paper chromatograms (Fig. 1).

Biochemical studies

Enzymic degradation. No enzymic hydrolysis of the methylated carbamoylcholines was observed with the bicarbonate method using high concentrations of purified preparations of butyrylcholinesterase, acetyl arylesterase and albumin from human serum. When these reaction mixtures were kept for 24 hr at 22 °C with subsequent paper chromatographic analyses of the mixtures, no degradation products were identified on the chromatograms. These same mixtures were tested pharmacologically after 2 weeks and showed the same activities as freshly prepared solutions. These results indicate that the N-methylated carbamoylcholines, as well as the unsubstituted compound known previously, are not hydrolysed by the three enzyme preparations tested.

Cholinesterase inhibition. The inhibitory effects of MeCbCh and Me_2CbCh on acetylcholinesterase of *Torpedo* electric organ and on butyrylcholinesterase of human serum are illustrated in Figs. 2 and 3; for comparison, the results obtained with unsubstituted carbamoylcholine and DCF are also included. The methylated choline esters were much stronger inhibitors than the unsubstituted ester, and less active than DCF, especially with serum cholinesterase. Choline, monomethylamine and dimethylamine had no inhibiting effect when used in the same concentration as those of the esters (1·0 mM) and under the same experimental conditions.

In the experiments with acetylcholinesterase (Fig. 2) the inhibitory effects of carbamoylcholine and DCF were almost independent of incubation time, i.e. the time during which the enzyme was incubated with the inhibitor before the substrate (acetylcholine) was added. Thus, there was little difference in the degree of inhibition obtained when the inhibitor was added to the enzyme before or at the same time as the substrate. DCF was a stronger inhibitor ($pI_{50} = 4$–$4{\cdot}5$) of this esterase than was carbamoylcholine ($pI_{50} < 3$).

Butyrylcholinesterase behaved differently towards carbamoylcholine and DCF, the reaction of the latter with this esterase being progressive. DCF was a much stronger

inhibitor than carbamoylcholine. A concentration of the substrate a hundred times higher than that of DCF was unable to reverse the esterase activity when added 6 min after the addition of the inhibitor (Fig. 3, *D*). There was little difference in the degree of inhibition obtained after 6 and 180 min incubation ($pI_{50} = 5{\cdot}5$). The reaction of DCF with human serum cholinesterase was therefore a comparatively rapid process of an irreversible type.

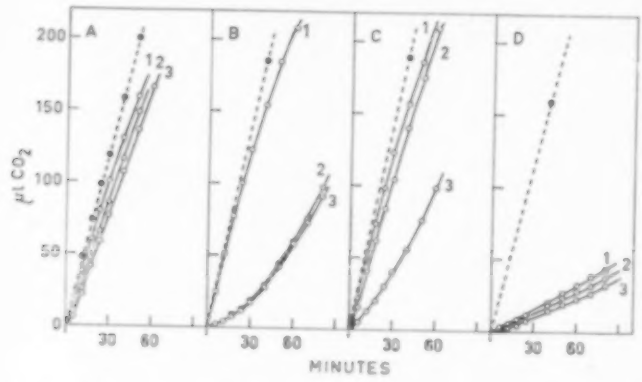

FIG. 2. Inhibition of acetylcholinesterase of electric organ by carbamoylcholines and DCF. Substrate, 10 mM acetylcholine chloride. Inhibitor, 1·0 mM carbamoylcholine chloride, its N-methyl derivatives, or DCF. Inhibitor-concentration values refer to those during esterase determination; the concentrations during enzyme-inhibitor incubation, before the addition of substrate, were five times higher. *A*: carbamoylcholine; *B*: MeCb Ch; *C*: Me_2CbCh; *D*: DCF; ●---●, control; (1) inhibitor and substrate simultaneously mixed with the enzyme; (2) and (3): enzyme incubated 6 and 180 min, respectively, with the inhibitor before the addition of substrate.

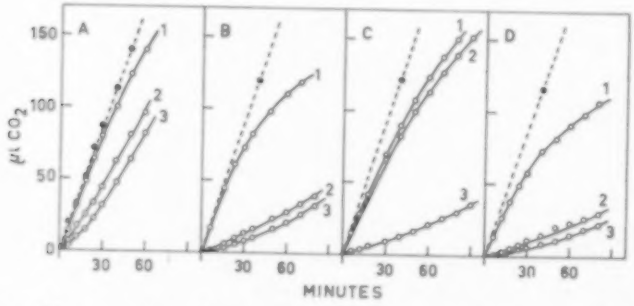

FIG. 3. Inhibition of human serum cholinesterase by carbamoylcholines and DCF. Experimental conditions and keys as in Fig. 3, except for the concentration of DCF, which was 0·01M.

The inhibitory properties of the methylated carbamoylcholines were intermediate between those of carbamoylcholine and DCF. The reaction rates with both acetyl- and butyrylcholinesterase were much higher for MeCbCh than for the Me_2CbCh. The inhibiting effects were stronger for butyrylcholinesterase than for acetylcholinesterase. After 180 min incubation, depending on the degree of inhibition, added substrate could reverse the esterase activity only partly or not at all.

These results indicate that the esterase inhibition by the methylated carbamoylcholines was irreversible. Dialysis of the reaction mixtures of esterase and MeCbCh also failed to reactivate the inhibited enzyme especially after long periods of incubation. In addition, esterase inhibition at various substrate concentrations, evaluated graphically in Fig. 4, was shown to be non-reversible. With reversible inhibition (competitive

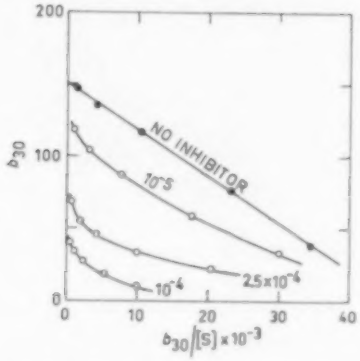

FIG. 4. Graphical evaluation of the inhibition of human serum cholinesterase by Me_2CbCh as function of substrate concentration. The esterase was incubated for 50 min with the inhibitor before the addition of acetylcholine. Esterase activities (b_{30}) were extrapolated values of hydrolysis rates 30 min after the addition of the substrate (S).

or non-competitive) these lines should be straight. The substituted carbamoylcholines probably reacted with the esteratic site of the enzyme molecule, because choline, in contrast to acetylcholine (Figs. 2 and 3), could not protect the enzyme from inhibiton, as was shown in an experiment (Table 2) with butyrylcholinesterase and MeCbCh.

TABLE 2. EFFECT OF CHOLINE ON THE CHOLINESTERASE INHIBITION BY MeCbCh

(Substrate, 10 mM acetylcholine chloride. Concentrations of choline chloride and MeCbCh during esterase determination, 10 mM and 2 mM, respectively. Controls performed in the absence of both choline and MeCbCh. In the presence of choline and MeCbCh the compound marked with an asterisk was added 6 min after the addition of the other. Activity values refer to b_{30} extrapolated from equilibrium curves.)

Additions	Acetylcholinesterase	Butyrylcholinesterase
Nil (control)	120	89
Choline	102	75
MeCbCh	28	7
Choline + MeCbCh	30	8
MeCbCh + choline	26	8

Pharmacological studies

Acute toxicity. The results of the toxicity determinations are summarized in Table 3.

Experiments in the anaesthetized cat

Effects on respiration. Both the monomethyl and the dimethyl analogue were powerful respiratory stimulants. The effect rapidly followed intravenous injection, and then

TABLE 3. ACUTE TOXICITY AS TESTED BY INTRAPERITONEAL INJECTION IN MICE

Compound	Acute toxicity (mg/kg)
Carbamoylcholine	3
MeCbCh	17
Me_2CbCh	17
DCF	0·17

rapidly faded (Figs. 5 and 6). The stimulation, which was apparent after doses of 10μg/kg body weight and was of about the same potency for both compounds, was often followed by a decrease in respiratory depth, sometimes apnea. The respiratory stimulation was sometimes proceeded by an apnea of short duration, as was most evident with MeCbCh, and coincided with an initial rapid decrease in blood pressure. The stimulation of the respiration appeared simultaneously with a rise in blood pressure (Fig. 5). The effects on the respiration had no evident relationship with the degree of neuromuscular block at the dose levels used. Clamping of the carotids, vagotomy, atropinization or administration of dibenamine did not essentially change the respiratory responses. Carbamoylcholine had no respiratory effects except in very high doses when some stimulation was also seen.

Effects on blood pressure and pulse rate. Carbamoylcholine had effects on blood pressure in doses from ten to fifteen times lower than the methylated compounds. At these low doses carbamoylcholine had a pure muscarinic effect on blood pressure and pulse rate, i.e. rapid fall and bradycardia (Fig. 7). These effects were completely abolished by atropine, when carbamoylcholinew as given in doses of 100μg/kg body weight. At this dose level no nicotinic effects could be demonstrated.

After low doses of both the monomethyl and the dimethyl analogue, i.e. up to about 10 μg/kg, the effects on blood pressure were predominantly muscarinic (Fig. 8). Higher doses gave essentially nicotinic effects of the usual biphasic type (Fig. 5). The monomethyl analogue, however, usually produced a rapid fall in blood pressure before the rise, in contrast to the dimethyl analogue (Fig. 5). Following atropinization both the compounds had nicotinic effects (Fig. 9), which were reversed by dibenamine, Vagotomy or clamping of the carotids did not essentially change the effects on blood pressure. Even high doses of the compounds did not alter the response to acetylcholine, given within about 8 min (Fig. 5).

Both the substituted analogues produced bradycardia after doses over 10 μg/kg (Fig. 8). At higher dose levels the initial bradycardia was usually followed by a transient tachycardia, and during the following period of normalization there were frequent extrasystoles particularly after Me_2CbCh (Figs. 5 and 6). Atropinization or vagotomia did not change this effect. Dibenamine, however, prevented the arythmia.

Effects on neuromuscular transmission. Both the monomethyl and the dimethyl analogue produced a transient neuromuscular block without evident initial potentiation after doses of about 100 μg/kg (Figs. 5 and 6). Carbamoylcholine also produced block at this dose level, but this was of a somewhat longer duration. Neither cumulation nor tachyphylaxia could be demonstrated after injections repeated at 15 min intervals.

Effects on nictitating membrane. Doses exceeding 20 μg/kg produced spontaneous

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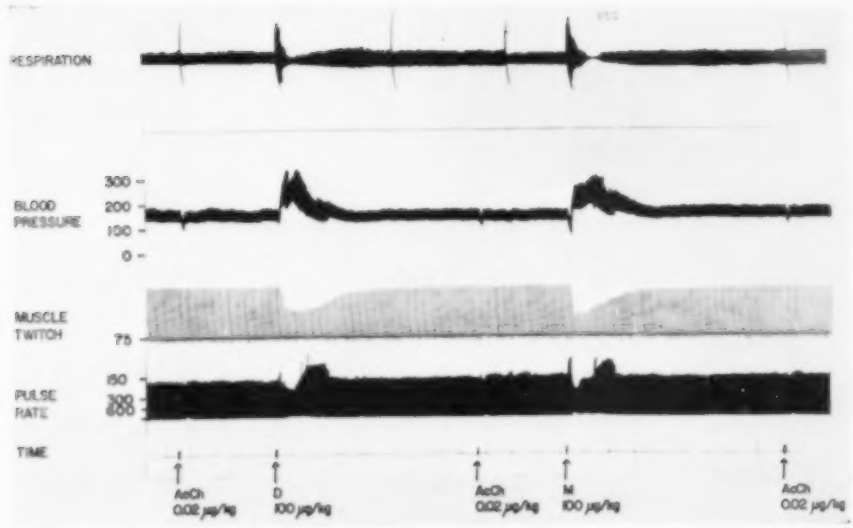

FIG. 5. Effects of MeCbCh (*M*) and Me_2CbCh (*D*) on respiration, blood pressure, neuromuscular transmission and pulse rate. Acetylcholine (AcCh) included for comparison. Time in minutes. Cat, 4·7 kg. Sodium pentobarbital anaesthesia.

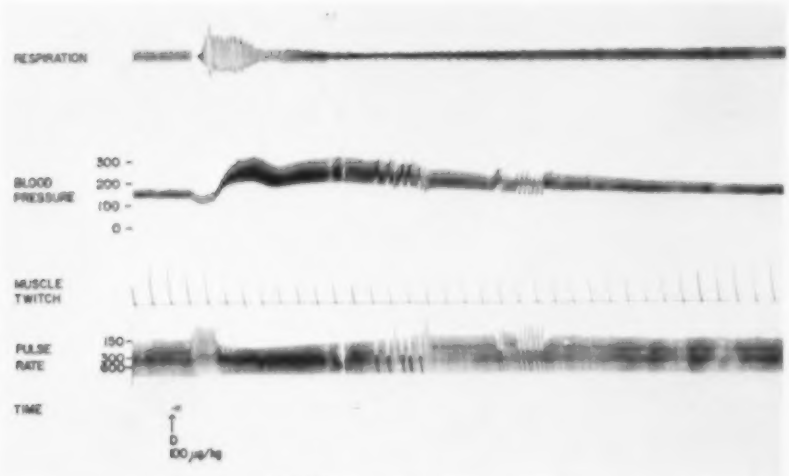

FIG. 6. Effects of Me_4CbCh (*D*) on respiration, blood pressure, neuromuscular transmission and pulse rate. Time in 10 sec. Cat 4·7 kg. Sodium pentobarbital anaesthesia.

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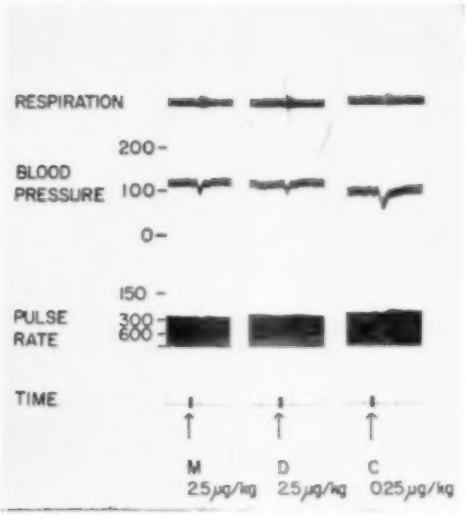

FIG. 7. Effects of MeCbCh (*M*), Me_2CbCh (*D*) and carbamoylcholine (*C*) on respiration, blood pressure and pulse rate. Time in minutes. Interval between injections 15 min. Cat 1·5 kg. Sodium pentobarbital anaesthesia.

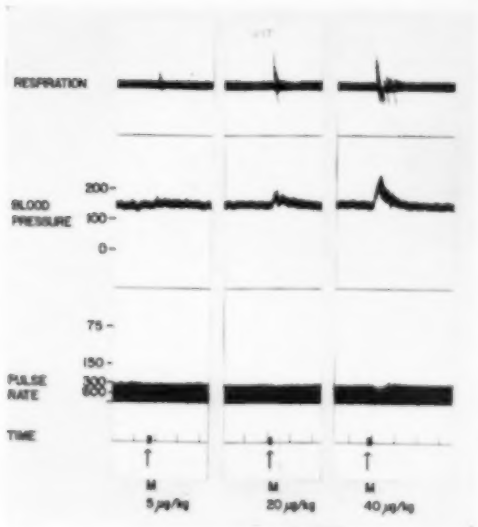

FIG. 9. Effects on respiration, blood pressure and pulse rate of increasing doses of MeCbCh (*M*) following atropinization (1 mg per kg). Time in minutes. Interval between injections 15 min. Cat 1·5 kg. Sodium pentobarbital anaesthesia.

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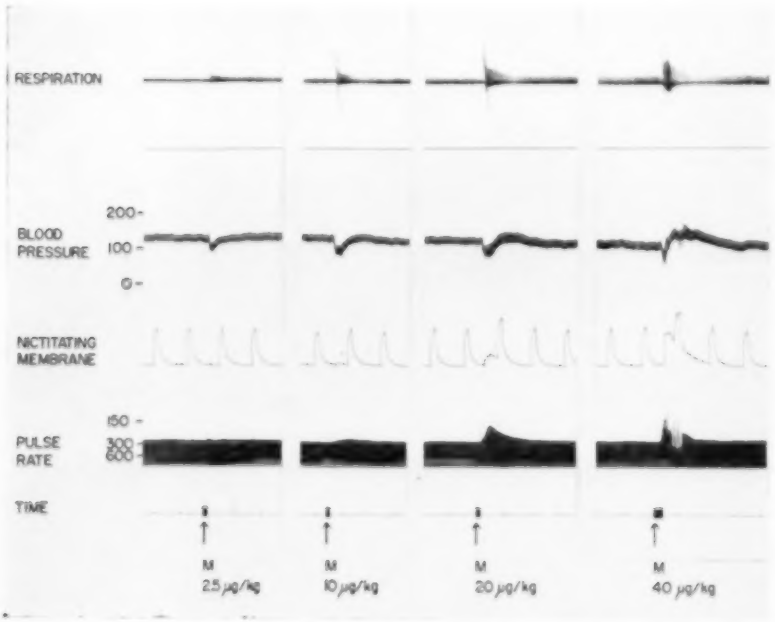

FIG. 8. Effects of increasing doses of MeCbCh (*M*) on respiration, blood pressure, nictitating membrane and pulse rate. Time in minutes. Interval between injections 15 min Cat 1·5 kg. Sodium pentobarbital anaesthesia.

contractions of the nictitating membrane and an increase in the response after stimulation of the sympathetic trunk (Fig. 8). Doses up to 100 μg/kg were tested, and no signs of blockade of the response to preganglionic stimulation were seen. The effects on the nictitating membrane were completely abolished by hexamethonium.

Duodenal intraluminal pressure. Carbamoylcholine produced an increase in the duodenal intraluminal pressure of considerably longer duration than did the substituted analogues, which also had a stimulating effect although slight.

Effect on femoral arterial blood flow. At doses exceeding 20 μg/kg i.v. the two substituted analogues produced an increase in femoral arterial blood flow, which coincided with the elevation of blood pressure and was followed by a decrease of short duration. When the blood pressure had reached a normal level, the blood flow was also usually normalized. Sometimes an initial decrease in blood flow was noticed. This appeared at the same time as the initial fall in blood pressure, and was most evident for the monomethyl derivative.

Effects on the isolated guinea-pig ileum and frog rectus. The results are summarized in Table 4. Carbamoylcholine was as potent as acetylcholine on the ileum, while the methylated compounds were much less potent. The effects on the guinea-pig ileum were completely abolished by atropine. On this preparation a bell-shaped dose–response curve was obtained for the two substituted analogues. On the frog rectus both the substituted derivatives were approximately four times more potent than carbamoylcholine and five times less potent than acetylcholine.

TABLE 4. RELATIVE MOLAR POTENCY (ACETYLCHOLINE = 100) OF CARBAMOYLCHOLINE AND THE TWO N-METHYLATED DERIVATIVES ON THE GUINEA-PIG ILEUM AND FROG RECTUS

Compound	Relative molar potency (Acetylcholine = 100)	
	Guinea-pig ileum	Frog rectus
Carbamoylcholine	100	5
MeCbCh	2·5	20
Me_2CbCh	0·7	20

DISCUSSION

The results of the pharmacological studies with the N-methylated carbamoylcholines presented in the present paper confirm previous observations[4] that substitution in the amino group of carbamoylcholine greatly reduces the muscarine-like activity. Me_2CbCh has almost pure nicotine-like activity and the monomethyl derivative is intermediate. The stimulating effect on respiration observed with the methylated derivatives was previously demonstrated with other choline esters.[15] The mechanism underlying this effect, however, has not been further investigated. Nor was it possible to explain the decrease in the response of the guinea-pig ileum produced by increasing doses of the methylated derivatives. Similar bell-shaped dose–response curves have been obtained with other quaternary ammonium compounds, and the phenomenon was recently discussed by van Rossum and Ariëns[16]. Possibly this decrease might be due to the reaction of the receptor with the carbamate moiety at high doses instead of with the choline radical of the molecule.

The methylated carbamoylcholines are moderate cholinesterase inhibitors and less active in this respect compared with the most active of known carbamates (e.g. physostigmine and the prostigmine analogues). The inhibitory mechanism is probably a carbamoylation of the active sites of the esterase molecule; the esteratic site is most likely to be involved. The results obtained with DCF[7,17] and a number of other N-alkyl and N:N-dialkylcarbamates[7] have indicated that the inhibition of cholinesterases by these agents involves the formation of carbamoyl derivatives of the esterases. The inhibitory process observed with the methylated carbamoylcholine is probably similar. Quantitative differences exist between various derivatives in this group of esterase inhibitors. The more easily the carbamate link can be broken by hydrolysis the more rapidly and strongly is the enzyme inhibited. The irreversible nature of this inhibitory reaction is analogous to the phosphorylation of esterases by organic phosphate esters.

In vivo no potentiation of the response to acetylcholine could be demonstrated with the methylated carbamoylcholines. This is probably due to the protective effect of the substrate (acetylcholine) on the esteratic site against inhibition (choline had no such effect), to the relatively low affinity of the compounds to the esterase, and to the fact that the reaction (carbamoylation) with the esterase is a comparatively slow process.

The biochemical studies indicated that the compounds studied are extremely stable in aqueous solutions and no esterase preparations tested were able to hydrolyse them at a measurable rate. However, the effects of the compounds *in vivo* are of comparatively short duration and no cumulative effect could be demonstrated. Plasma esterases are only occasionally involved in the hydrolysis of these compounds, as has also been noticed previously with other carbamates.[7,8] Other systems must be involved in the detoxication, the nature of which is not known.

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SHORT COMMUNICATIONS

The effect of chlorpromazine on the aconitase in kidney particle preparations

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In the course of some other work, the writer tried the effect of chlorpromazine on the metabolism of kidney particles from the guinea pig. The experiments were arranged so that in 30 min the particles almost completely metabolized 10 μmole citrate, added at the start of the experiment. Figure 1, compiled from four experiments, shows that addition of chlorpromazine stopped citrate metabolism; $4{\cdot}7 \times 10^{-4}$M had an effect over 50 per cent, whereas $1{\cdot}88 \times 10^{-4}$M did nothing. Many of the biochemical observations made in this field appear to be rather unspecific, though recent observations on cytochrome oxidase[1] are more definite. One possible explanation of the aconitase results is that the chlorpromazine forms a non-ionized compound with the citrate which then becomes inaccessible to the enzyme. The steepness of the curve would be consistent with this. If this be the explanation, observations upon compounds formed between ATP and chlorpromazine and other substrates may show similar complexing effects. This may well explain the lack of specificity in action upon mitochondrial reactions which has been reported, for instance, by Berger.[2]

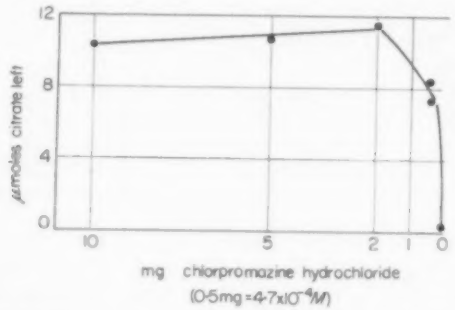

Fig. 1. Relation between concentration of chlorpromazine and the disappearance of added citrate with kidney particles.

Experiment: Kidney particles (guinea pig) were homogenized by our usual methods,[3] centrifuged and made up in 1 % KCl containing 0·1 M phosphate buffer, pH 7·2, so that the particles from one guinea pig made up a volume of 26 ml. Flasks for experiment contained 1·9 ml enzyme suspension Mg^{2+} 0·1 ml (4 μmoles), ATP 0·1 ml (1·2 μmoles) Na citrate 10 μmoles, chlorpromazine hydrochloride (neutralized) and enough 1 % KCl to bring the total volume to 3·0 ml. The reaction was run for 30 min at 37 °C, after which trichloracetic acid was added to 8 per cent. Citrate was determined by Taylor's[4] method. Chlorpromazine hydrochloride was "Largactil".

Acknowledgement—I am indebted to Mrs. R. Jourdan (Shawdon) for valuable technical assistance.

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The fate of ^{14}C-labelled iproniazid in the rat*

(*Received* 11 *August* 1959)

IT HAS been reported by Hess *et al.*[1] that monoamine oxidase inhibition produced by iproniazid (1-*iso*nicotinyl-2-*iso*propylhydrazine) persists for 5 to 6 days after measurable (by chemical methods) quantities of the drug have disappeared from the tissues. The inhibition produced has been shown to be of an irreversible type, both *in vitro* and *in vivo*,[2] and Davison[3] has proposed a mechanism involving the whole molecule mediated through the dehydrogenated product. The complex proposed by Davison retained the *iso*nicotinoyl moiety of iproniazid and it was thought that iproniazid labelled at the C=O group would be a good tool to confirm this. Accordingly, we have synthesised ^{14}C-labelled iproniazid† with the label at the C=O group and studied its distribution pattern in the rat. Our results confirm the finding of earlier workers that *iso*nicotinic acid is the major metabolite of iproniazid and that the drug or its metabolite, *iso*nicotinic acid, is not retained in the blood, brain, or liver. Almost all of the injected radioactivity has been recovered, as iproniazid and *iso*nicotinic acid, in the urine collected during the subsequent 24 hours. An explanation is suggested for the persistence of inhibitory activity in the light of these data.

METHODS AND RESULTS

Male white rats weighing 300–350 g were given 25 mg of radioactive iproniazid per kg intraperitoneally ($0{\cdot}92 \times 10^6$ dis/min per mg) and, at the end of definite intervals of time, blood samples were taken by cardiac puncture. The animals were then sacrificed and the brain and liver removed quickly, washed and chilled. In one case the brain was perfused with physiological saline. All animals were anesthetised with pentobarbital, and heparin was given prior to sacrifice. There was no significant difference in the amount of radioactivity found in the brain between the perfused and the non-perfused animal. Urine collected from the 24 hr experiment and the homogenates of the brain and liver were assayed for radioactivity and then fractionated. The fractionation process consisted of a preliminary extraction with ether followed by extraction with chloroform (at pH 7·5–8). The chloroform extractives, after removal of the solvent, were taken up in water and chromatographed on paper. The aqueous residue from chloroform extraction was also chromatographed on the same sheet of paper. The paper had three slits 2 mm wide extending longitudinally from just above the line where the test material is applied to about 2 cm from the bottom of the paper. This facilitated application of the chloroform extract, the aqueous residue, and authentic samples of iproniazid and *iso*nicotinic acid

TABLE 1. TISSUE DISTRIBUTION OF RADIOACTIVITY FROM ^{14}C-LABELLED IPRONIAZID‡ (EACH VALUE IS A MEAN OF FOUR EXPERIMENTS)

Time	Radioactivity (in dis/min per mg dry wt)		
	Plasma (dis/min per ml)	Brain	Liver
10 min	23,170	78	48
20 min	43,600	102	135
1 hr	29,650	83	96
24 hr	0	0	0
			in this case about 98 per cent the total injected radioactivity was recovered in the urine

‡ Chromatography and autoradiography of the extracts of these tissues, followed by treatment with cyanogen bromide and benzidine, showed the presence of two spots identified as *iso*nicotinic acid and iproniazid (by comparison with authentic samples). Quantitative estimations were not done since these studies were primarily directed towards obtaining information concerning the maximum period the drug or its metabolites are retained in the brain.

* This investigation was supported in part by a research contract No. AT-(11-1)45 between the United States Atomic Energy Commission and the University of Chicago, and the Wallace C. Abbott Memorial Grant.

†The synthesis will be reported elsewhere.

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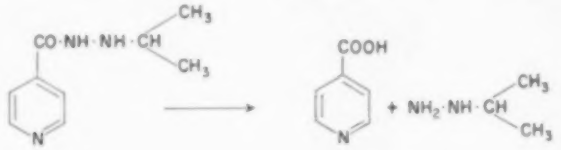
on the same sheet of paper without possible contamination. After development with *n*-propanol–water (4:1), the chromatograms were exposed to Kodak no-screen X-ray film and were subsequently treated with cyanogen bromide and benzidine.[4] The zone of radioactivity in the autoradiogram corresponded with the color zone produced by cyanogen bromide–benzidine. Iproniazid was detected in the chloroform extract, and *iso*nicotinic acid in the aqueous residue. About 98 per cent of the total injected radioactivity was recovered, as iproniazid and *iso*nicotinic acid, in the urine collected during the 24 hr period. The results are summarised in Table 1.

DISCUSSION

It will be seen from Table 1 that the radioactivity reaches a maximum in the brain within an hour and disappears completely at the end of 24 hr. Liver and plasma also show a similar pattern. Analysis of the tissue extracts showed that the contribution to the radioactivity came from both iproniazid and *iso*nicotinic acid. At the end of a 24 hr period no trace of iproniazid or *iso*nicotinic acid could be detected in tissues, either by radio-assay or by chemical tests (CNBr–benzidine). These findings are essentially in agreement with those of Hess *et al.*,[1] using non-radioactive iproniazid, except that in the plasma they were able to detect trace quantities of the drug (less than 10 μg/ml) 48 hr after injection. It is not possible to say whether this is due to the difference in the methods of assay or whether this is related to the high dose of iproniazid used by Hess *et al.* in their studies (195 mg/kg, cf. LD_{50} 117 mg/kg). The results of these studies indicate that neither iproniazid nor *iso*nicotinic acid is stored in the brain, liver or blood. However, it can be seen that *iso*propyl hydrazine (b), or a metabolite of it, if



(a) (b)

retained in the tissues will not be detected by the methods used in the present studies or by those used by Hess *et al.* At the dose level of iproniazid we have used, it was not possible to detect *iso*propyl hydrazine or its derivatives in the tissues by ordinary chemical methods. It is known that alkyl hydrazines are much more toxic to the tissues than hydrazine. If the *iso*propyl hydrazine that is liberated in the brain by hydrolytic cleavage causes alteration or destruction of the sites of enzyme action or enzyme synthesis (amine oxidase), it is conceivable that the amine oxidase inhibitory power attributed to iproniazid is due to this effect and thus persists long after the drug or its metabolite has disappeared. When this damage is repaired, the tissue returns to its normal activity. Injuries to areas other than the amine oxidase system are not excluded by this hypothesis. A similar situation in the liver could explain the pathological changes (indistinguishable from viral hepatitis) observed in the liver of human subjects who exhibited the toxic effects of iproniazid. The fact that *iso*nicotinic acid hydrazide does not cause any effects comparable to those of the *iso*propyl derivative is also consistent with this reasoning. The recent finding of Koechlin and Iliev[5] that tissues retain radioactivity for longer periods with iproniazid labelled at the C_2-*iso*propyl group adds interest to this problem. Previous work of Roth[6] and Barlow[7] emphasizes the uniqueness of drug entry and distribution in the central nervous system. Autoradiographic studies with the labelled drug designed to show any discrete anatomical distribution, in combination with discrete biochemical distribution and histochemical analysis, should provide additional information regarding the mechanism of action of iproniazid. Such experiments are in progress.

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BOOK REVIEWS

J. GUY: **Mathématiques préparatoires aux sciences expérimentales.** (Mathematics in preparation for experimental sciences). S.E.D.E.S., Paris, 1959. pp. 306., 2.100 French francs, 252 Belgian francs.

THIS book is mainly intended for students of pharmacy, medicine and the sciences, to help their understanding of physics and chemistry, but it is also likely to be extremely useful to research workers wishing to improve their basic knowledge of mathematics.

It contains numerous practical examples, is presented in an essentially graphical manner, and should enable the attentive reader to manipulate the vectorial calculation, ordinary integrals and differential linear equations, even when the basic knowledge of the reader does not go beyond general certificate of education standard.

Z. M. BACQ

Eléments de calcul des probabilités et de calcul statistique. (Basic elements of the theory of probabilities and of statistical calculation). C.D.U., Paris, 1959. pp. 170, 850 French francs, 105 Belgian francs.

THIS booklet should be very useful to all biologists. After a simple but relatively comprehensive study of the principle theorems of the theory of probabilities (probability of causes, theory of deviations, correlation, laws of Gauss and of Poisson, etc.), the author examines the leading ideas of statistical calculation. In particular, the last two chapters concern the techniques of descriptive analysis and various examples of interpretation are fully treated (tests of hypotheses, significant difference and methods of estimation of parameters).

Z. M. BACQ

ANDREW WILSON and H. O. SCHILD: **Applied Pharmacology** (CLARK), 9th Edition. J. and A. Churchill, Ltd., 1959. 750 pp. 50s.

A. J. CLARK wrote the first edition of this book in 1923 to demonstrate the importance of scientific evidence in the clinical application of drugs. It helped to bridge the gap between pharmacology and therapeutics and has been popular with medical students ever since that time. The 7th edition appeared in 1940 and was the last for which CLARK was responsible. ANDREW WILSON and HEINZ SCHILD have prepared the 8th and 9th editions, which appeared in 1952 and 1959. Pharmacology has advanced rapidly, and the book has grown, in this last edition, from 690 to 750 pages; the introduction of 65 new figures has raised the total number from 120 to 165, so that 20 figures must have been discarded; there is a remarkable picture drawn under the influence of LSD (lysergic acid diethylamide).

This book provides a readable general account of those aspects of pharmacology which are of interest to medicine. It is up to date and reliable and contains general references for further reading, and lists of the more important official preparations of drugs with their doses.

The 9th edition contains a number of new features which improve the value of the book. The chapter on the general principles of drug action has five new pages dealing with bioassays and clinical trials, which partly fill a gap present in the older editions. There are new pages about chemical transmitters and about hormones. The chapter on inorganic metabolism has been omitted. The chapter on the functions of the central nervous system has been replaced by new and valuable chapters dealing with methods of testing the effects of drugs on the mind (by Dr. HANNAH STEINBERG). There is a valuable account of the pharmacology of LSD, which causes temporary insanity, chlorpromazine reserpine and

meprobamate which are used in the treatment of insanity, of drugs which banish boredom and drugs which banish fear. The chapters dealing with the blood, the kidneys, and chemotherapy have all increased in length.

Errors and omissions are few and unimportant. The molecular weight of insulin is given as 48,000; adrenaline is said to be excreted as a sulphate (there is no evidence for this) the release of 5-hydroxytryptamine by reserpine is mentioned, but not that of noradrenaline, which may well be more important. These minor faults do not spoil the value of the book and are only mentioned to show that the reviewer has studied the text. The authors are to be congratulated on a good piece of work.

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Papers to be published in future issues

J. S. LOWE: Metabolism of compounds related to ethyl mercaptan.

R. A. DEITRICH and D. L. SMITH: Effect of the thyroid on butyrate oxidation in the rat heart.

K. F. NAKKEN, L. ELDJARN and A. PIHL: The mechanism of inactivation of penicillin by cysteine and other mercaptoamines.

P. BARBIERI, A. DIMARCO, L. FUOCO and A. RUSCONI: Investigations on the mode of action of *cyclo*serine upon protein synthesis in *Escheicha coli*.

V. C. DEWEY, M. R. HEINRICH, D. G. MARKEES and G. W. KIDDER: Multiple inhibition by 6-methylpurine.

W. H. PRUSOFF, J. J. JAFFE and H. GUNTHER: Studies in the mouse of the pharmacology of 5-iododeoxyuridine, an analogue of thymidine.

W. WELLING, J. A. COHEN and W. BERENDS: Disturbance of oxidative phosphorylation by an antibioticum produced by *Pseudomonas cocovenenans*.

K. VAN ASPEREN: Toxic action of organophosphorus compounds and esterase inhibition in houseflies.

R. W. IRWIN and H. J. SMITH—III. Cholinesterase inhibition by galanthamine and lycoramine.

IRVING I. A. TABACHNICK: The metabolism of gamma-aminobutyrylcholine.

P. ROSENBERG: Reaction *in vivo* by PAM of brain cholinesterase inhibited by Paraoxon.

L. MIKKONEN, T. TUOMINEN and E. KULONEN: Collagen fractions in lathyritic rats.

A. V. MARTON and W. KALOW: Inhibitors of aromatic esterase of human serum.

T. FREDRIKSSON and G. TIBBLING: Inhibition of cholinesterase with methylfluorophosphorylcholine and -carbocholine. Spontaneous return of activity.

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EFFECT OF THE THYROID ON BUTYRATE OXIDATION IN THE RAT HEART*

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(*Received* 7 *July* 1959)

Abstract—Oxidation of butyrate by rat heart homogenates in the presence of malate, nicotinamide or endogenous "sparkers" has been studied. It was found that heart homogenates from hyperthyroid rats oxidized butyrate at a higher rate than those from normal or hypothyroid animals. It is suggested that these findings may have some bearings on the mechanisms of cardiac abnormalities associated with thyroid dysfunction.

IT HAS been recognized for many years that the thyroid has a profound effect on cardiac function. The hyperthyroid individual exhibits tachycardia and an increased cardiac output[1] terminating eventually in high output failure.[2] In hypothyroidism the heart is subject to low output failure.[3] The causes of these effects are unknown. In recent years much work has been done in the area of *in vitro* metabolism of the heart in search of a possible explanation for the *in vivo* manifestations of altered thyroid function. The work of Gemmill,[4] Ullrick and Whitehorn[5] and Barker[6] are examples. The demonstration by Bing *et al.*[7] and Gordon[8] that the heart normally utilizes fatty acids for a large portion of its energy requirements has focused our attention on the oxidation of fatty acids in the heart as a possible point of attack on the problem of relating thyroid disease to altered cardiac function.

Olsen and Piatnek[9], employing cardiac catheterization, have shown that the hearts of thyrotoxic dogs extracts a greater amount of fatty acids from the blood than do the hearts of euthyroid animals. It appeared to be possible that the ability of the thyrotoxic heart to use fatty acids in excess would support the increased cardiac work. Conversely, if the hypothyroid heart oxidized fatty acids in amounts less than normal, a partial explanation of low output failure in hypothyroidism would be possible.

EXPERIMENTAL

Young rats (130–150 g) of the Denver strain were used. They were made hyperthyroid by injection of 200 μg of Na-L-thyroxine pentahydrate‡ per g, subcutaneously, once daily for two weeks. Hypothyroidism was induced by surgical thyroidectomy, injection of I^{131} intraperitoneally, or by administration *ad libitum* of 0·1 per cent thiouracil in the drinking water. Surgically thyroidectomized animals were provided with 1 per cent calcium lactate in the drinking water for one week and sacrificed 14 days postoperatively. I^{131} was injected in a dose of one mc per rat. These animals were used approximately 30 days after injection. Thiouracil was given in the drinking

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† American Foundation for Pharmaceutical Education Fellow.
‡ Generously supplied by the Smith Kline and French Co., Philadelphia, Pa.

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water for 14 days prior to experimentation. The basic metabolic rate of the animals was determined by a modification of the method of d'Amour and Blood[10]. Conventional manometric techniques were used to determine butyrate oxidation. The animal was stunned by a blow on the head, the heart immediately removed and placed in ice-cold 0·25 M sucrose. Only the ventricles and intraventricular septum were used. The tissues were blotted dry, weighed and placed in a Tenbrock glass homogenizer containing enough ice-cold homogenizing fluid to yield 80 mg of tissue per ml. The homogenizing medium consisted of 80 parts of 0·25 M sucrose and 20 parts of 0·05 M potassium phosphate buffer, pH 7·4. The heart was rapidly homogenized by a combination of hand and motor driven homogenization with a loose-fitting pestle. The homogenate was strained through two layers of gauze to remove any pieces of connective tissue. Of the homogenate, 0·5 ml was pipetted into ice-cold Warburg flasks containing the reaction mixture described by Plaut and Plaut,[11] except that in some flasks 0·5 μmole of malate was replaced by 15·0 μmole of nicotinamide or water. Butyrate was present in a final concentration of 0·001 M (1·5 μmole per flask). The final volume was 1·5 ml in the main compartment and the center well contained 0·1 ml of 10 per cent KOH. All solutions except nicotinamide were adjusted to pH 7·4 with KOH. The flasks were placed in the water bath at 30 °C, and, after an equilibration period of 10 min the stopcocks were closed and readings taken every 10 min for one hr. A final reading was taken at 90 min. In all experiments, the homogenate was ice-cold during preparation and no more than 15 min elapsed between killing the animal and placing the flasks in the water bath. Deviation from this procedure resulted in inactive preparations.

RESULTS AND DISCUSSION

All results are corrected for control flasks containing no butyrate and are expressed as μl. of oxygen uptake per 40 mg of heart tissue, wet weight. The control flasks containing no butyrate usually had an oxygen uptake of about one-half that obtained when butyrate was added. When duplicate flasks were employed, the values were averaged and treated as one value for the statistical analysis.

TABLE 1. O_2 UPTAKE IN mm^3 DUE TO ADDED BUTYRATE BY 40 mg OF HOMOGENIZED RAT HEART VENTRICLES

		Additions to Flasks											
		Butyrate				Malate Plus Butyrate				Nicotinamide Plus Butyrate			
Group	BMR*	40 min	S.E.	90 min	S.E.	40 min	S.E.	90 min	S.E.	40 min	S.E.	90 min	S.E.
Control	2·67	15·0† (11)	±2·36	21·6 (11)	±2·88	15·7 (16)	±2·51	27·4 (16)	±3·73	49·0 (21)	±4·10	65·3 (21)	±4·13
Hyper-thyroid	3·47‡	76·3 (12)	±1·92	112·2‡ (12)	±2·89	91·0‡ (14)	±1·96	131·4‡ (14)	±2·35	70·6‡ (14)	±3·40	123·2‡ (14)	±5·86
I^{131} Treated	1·73‡	7·1 (16)	±1·50	10·2‡ (16)	±1·94	9·9 (15)	±1·70	16·9 (15)	±2·40	24·3‡ (16)	±3·80	43·9 (16)	±7·30
Thyroid-ectomized	1·60‡					9·3 (16)	±2·14	11·9‡ (16)	±3·01	12·5‡ (16	±2·82	18·5‡ (16	±3·73
Thio-uracil treated	1·86‡	7·9 (4)	±1·38	10·5 (4)	±4·19	7·8 (9)	±1·49	12·3‡ (9)	±2·09	22·3‡ (9)	±2·81	30·6‡ (9)	±2·96

* Basal metabolic rate, ml of oxygen consumed at standard conditions/min per 100 g of rat.
† Numbers in parenthesis indicate the number of animals used.
‡ Significantly different from control, $p < 0{\cdot}001$.

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As can be seen from Table 1, oxygen uptake due to added butyrate in heart homogenates obtained from euthyroid animals was considerably increased by addition of nicotinamide. Presumably the increased oxygen uptake in the presence of nicotinamide is attributable to maintenance of functional diphosphopyridine nucleotide (DPN) since this is a well-known effect of nicotinamide.[12] Functional DPN then would be a limiting factor in the systems not containing nicotinamide. Addition of DPN to normal heart homogenates results in greatly increased total oxygen uptake, very little of which could be ascribed to oxidation of butyrate. Similar results are reported by Allen *et al.*,[13] except that these investigators observed increased $C^{14}O_2$-production from C^{14}-labelled butyrate when DPN was added. This failure of added DPN to stimulate the oxygen uptake due to butyrate illustrates the importance of binding of DPN.[14, 15] Addition of malate to heart homogenates from euthyroid animals resulted in very little increase in oxygen uptake due to butyrate. This is explained by the probability that there are enough oxaloacetate or oxaloacetate precursors present endogenously to "spark" the oxidation of butyrate.

Oxygen uptake due to butyrate was diminished in the heart homogenates obtained from hypothyroid animals in all systems employed. The reduction of the butyrate oxidation in the nicotinamide-protected system was the most consistent finding. Since one possibility is that nicotinamide is acting by protecting functional DPN, these observations may indicate a decreased amount of functional DPN in these homogenates. Because the amounts of coenzyme A,[16] cytochrome-*c* [17] and phosphate acceptors,[18] are decreased in hypothyroid tissues, these substances were added to the flasks in various experiments in attempts to raise the lowered values toward normal. No increase in oxygen uptake due to butyrate was observed when any one of these substances was added. In addition, flavine adenine dinucleotide, lipoic acid and L-triiodothyronine were added to heart homogenates from hypothyroid animals in separate experiments without any increase in butyrate oxidation.

In heart homogenates obtained from hyperthyroid animals, the oxygen uptake due to butyrate was increased to a marked degree. It is interesting that the oxidation in the nicotinamide-protected system was no greater than that measured in the malate and endogenous systems; therefore, it is possible that functional DPN is not rate-limiting in the heart homogenate from hyperthyroid animals. Very little information is available in the literature concerning the amounts of functional DPN in heart tissue obtained from hypothyroid or hyperthyroid animals. There are several reports of decreased total pyridine nucleotide concentrations in livers of hyperthyroid animals.[18, 19] It is evident from our results that the total pyridine nucleotide may not be the important consideration, but rather that the amount of functional DPN may be the crucial factor.

If the increased ability to oxidize butyrate, shown by the hyperthyroid heart in our experiments, were to extend to longer chain fatty acids, it is possible that the hyperthyroid heart would be able, on this basis, to support a significantly increased work load. The hyperthyroid heart, less able to oxidize fat, would not support a large increase in work load and would be subject to low output failure.

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THE MECHANISM OF INACTIVATION OF PENICILLIN BY CYSTEINE AND OTHER MERCAPTOAMINES

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Abstract—The kinetics of penicillin inactivation by various mercaptoamines have been followed by polarimetry. In the case of primary mercaptoamines the reactions are of second order with respect to time and concentration of the reactants. By determining the appropriate rate constants under different conditions of pH and ionic strength the conclusion is reached that the rate limiting step is a bimolecular reaction involving the mercaptide ion. The mechanism probably involves the formation of a semimercaptol. Several possible mechanisms for the role of the amino group in the opening of the β-lactam ring are discussed. The inactivation of penicillin by the tertiary mercaptoamine N:N-dimethylcysteamine nearly follows first order kinetics and gives as end product a partially racemized penicilloic acid.

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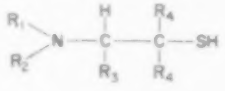
THE early discovery that penicillin is rapidly inactivated by cysteine and related mercaptoamines with opening of its β-lactam ring[1] focused interest on the reactivity of this structure, and it has been widely assumed that the specific binding of penicillin to sensitive bacteria and its antibiotic action involves a reaction of the β-lactam ring.[2]

The details of the interaction of mercaptoamines and amines with the β-lactam ring of penicillin are still inadequately understood. In the early inactivation studies which have been thoroughly reviewed by Wintersteiner *et al.*[3] only qualitative evaluations were carried out. This work permitted a classification of the compounds tested into three groups: "Fast inactivators", "slow inactivators", and compounds exerting no measurable effect. The 1:2-mercaptoamines were found to be "fast penicillin inactivators". It was established that the activity of the fast inactivators is dependent on the presence of a free sulphydryl group and a basic amine group on the adjacent carbon atom. The influence of substituents on the inactivating ability of 1:2-mercaptoamines may be summarized as follows:



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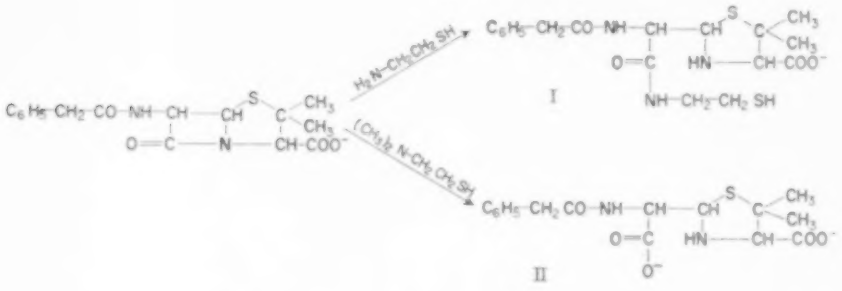
Inactive	*Active*
R_1 = Acyl	R_1 = H or Alkyl
R_2 = Alkyl	R_2 = H or Alkyl
R_4 = Alkyl	R_3 = H, COO^- or COOR
	R_4 = H

* Fellow of Landsforeningen mot Kreft (The Norwegian Cancer Society).

With primary mercaptoamines the overall reaction product was found to be the N-penicilloyl amides (I), whereas with tertiary amines such as N:N-dimethylcysteamine, the corresponding reaction product was the free penicilloic acid (II).



It is thus quite clear that these reactions involve an attack of the inactivator on the carbonyl carbon atom of the β-lactam ring with a rupture of the amide linkage.

In the present paper kinetic data are presented on the inactivation of penicillin by various mercaptoamines and amines. Use has been made of the fact that the opening of the β-lactam ring can be followed by measuring the decline of the optical rotation of the penicillin salt.[3] A mechanism is proposed for the reaction of penicillin with primary and secondary β-mercaptoamines, a mechanism which may have a bearing on the antibiotic activity of penicillin.

EXPERIMENTAL

Materials. The sodium benzylpenicillinate used was a gift from Apothekernes Laboratorium, Oslo. The specific rotation $[\alpha]_D^{23}$ of a 10^{-2} M solution in 0·5 M phosphate buffer at pH 7·4 was +288°. N:N-Dimethylcysteamine and N:N-diethylcysteamine were generously supplied by Deutsche Gold- und Silber-Scheideanstalt, Frankfurt a.M.; L(−)Cysteine hydrochloride was obtained from Hoffmann-La Roche & Co., Basel; D(+)cysteine from Fluka AG. Chemische Fabrik Buchs/SG; L(−)cysteine ethyl ester from Nutritional Biochemicals Corp., Cleveland; L(−)cysteinylglycine from Schwarz Laboratories, Mount Vernon; D(−)penicillamine·HCl·1/2 H_2O from California Foundation for Biochemical Research, Los Angeles; and hydroxylamine hydrochloride from Hopkin & Williams, Ltd., London. Penicillinase was purchased from Schenley Laboratories Inc., New York.

Cysteamine was prepared according to Gabriel *et al.*[4] and aminoethyl*iso*thiouroniumbromide hydrobromide (AET) was synthesized according to the method of Clinton *et al.*[5]

Polarimetric measurements. The reactions were carried out in strong (0·5 M) oxygen-free phosphate buffer in order to counteract the fall in pH during the course of the reaction. The buffer contained 0·02 M disodium ethylenediaminetetra-acetate (EDTA) in order to prevent oxidation of the thiols. The buffer was prepared from doubly distilled water and the solid components were dissolved under a stream of oxygen-free nitrogen. The reactions were started by the addition of penicillin. The polarimeter

tube (20 cm length) was filled under a stream of nitrogen. Readings were taken every minute. The fraction of penicillin which had reacted at any time was estimated from the formula

$$f = \frac{\alpha_0 - \alpha_t}{\alpha_0 - \alpha_\infty}$$

where α_0 is equal to the initial rotation, α_t is the rotation observed at the time t, and α_∞ is the final rotation observed in the reaction mixture. The initial rotation of the reaction mixture was calculated from the specific rotation of penicillin. When D(+)- or L(−)cysteine was used as inactivators, allowance was made for the optical rotation of these compounds. The necessary correction factors varied from 0·98 to 1·02.

Hydroxamic acid was measured according to Lipmann and Tuttle.[6]

RESULTS

The present kinetic measurements are based on the fact that the opening of the β-lactam ring of penicillin is associated with a change in optical rotation. The data here presented will therefore reflect the overall rate of the reaction sequence leading to the rupture of the amide linkage. In Fig. 1 typical data are presented.

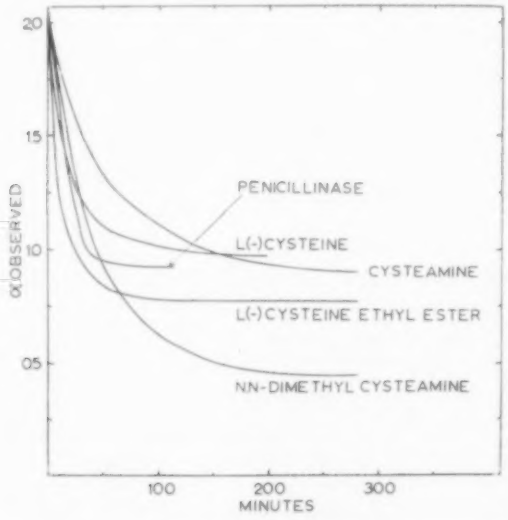
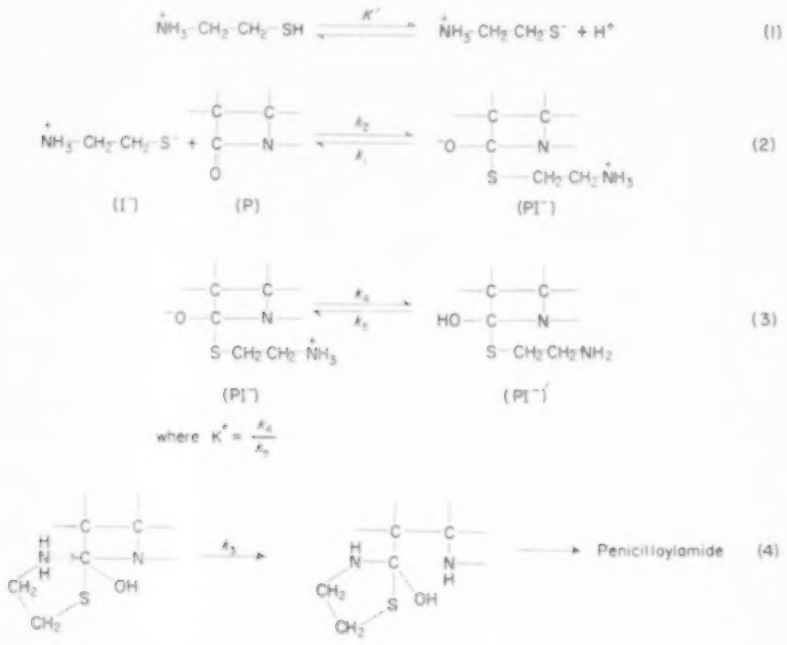


FIG. 1. The inactivation of penicillin followed by polarimetry. The concentration of penicillin was 10^{-2} M and that of the mercaptoamines $1{\cdot}5 \times 10^{-2}$ M, except for N-dimethylcysteamine, which was 3×10^{-2} M. Penicillinase (1000 Schenley units) was incubated with $2{\cdot}5 \times 10^{-3}$ M penicillin. The α plotted in this case is equal to observed α × 4.

The assumed reaction mechanism

In order to facilitate the subsequent discussion it is proposed to present at this time the reaction mechanism which in our view is considered the most likely one. The reactions are believed to proceed by way of the semimercaptol and the C_2-hydroxythiazolidine to give the penicilloylamide (reactions 1–4). This reaction mechanism has features in common with the thiazolidine mechanism proposed by Woodward *et al.* (see ref. 3). On the basis of previous work in this laboratory on the interaction of

cysteamine with aldehydes[8] to form thiazolidines, it is believed that the semimercaptol formation (reaction 2) is the rate limiting step.



Whereas reactions 1–3 are reasonably well established by our data, reaction 4 is open to discussion.

In the above scheme reactions 1 and 3 undoubtedly are almost instantaneous.

If it is assumed that the semimercaptol is present in small and constant amounts during the reaction (steady state), the rate constant k for the overall reaction can be shown to be:

$$k = \frac{k_2 \cdot k_3 \cdot K''}{k_1 + k_3\left(\frac{k_1}{k_5} + K''\right)} = \frac{k_2}{\frac{k_1}{k_3 K''} + \frac{k_1}{k_4} + 1} \tag{5}$$

If $k_3 \cdot K'' \gg k_1$ and if $k_4 \gg k_1$, k equals k_2. The reaction will then obey the following equation:

$$-\frac{dP}{dt} = k_2(I^-)(P) \tag{6}$$

It follows from equation 1 that:

$$(I^-) = \frac{(I_{\text{total}})}{1 + 10^{pK - pH}} \tag{7}$$

If I_{total} is substituted for I^- in equation 6, equation 8 is obtained:

$$-\frac{dP}{dt} = k_p(I_{\text{total}})(P) \tag{8}$$

where k_p is the practical rate constant. Clearly,

$$k = k_p(1 + 10^{pK-pH}) \tag{9}$$

The determination of rate constants

It follows from equation 6 and 8 that the assumed reaction mechanism requires that the inactivation of penicillin by cysteamine be second order both with respect to time and the concentration of the reactants. When the inactivation of penicillin by primary mercaptoamines was plotted as second order reactions with respect to time, straight lines were obtained up to the point where about 40 to 50 per cent of the inactivator had reacted (Fig. 2). This was the case irrespective of the concentration of the react-

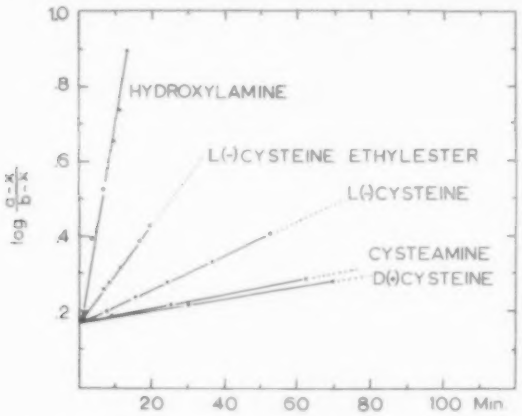


FIG. 2. Second order rate plots of penicillin inactivation by various mercaptoamines and amines. Conditions: pH 7·05, temperature 23 °C, penicillin concentration 10^{-2} M, inactivator concentration $1{\cdot}5 \times 10^{-2}$ M.

ants when L(−)- and D(+) cysteine, cysteamine, L(−)cysteine ethyl ester, L(−)-cysteinylglycine, and penicillamine were used as inactivators. The deviation from second order kinetics in the last part of the reaction, may be attributed to a small but unavoidable drop in the pH of the solution. It was concluded that the reactions were indeed of second order and the rate constants were calculated from the equation:

$$k_p = \frac{2{\cdot}303}{(a-b)t} \log \frac{b(a-x)}{a(b-x)} \tag{10}$$

where a and b are the initial concentration of the inactivator and of penicillin, respectively, and x is the number of moles of penicillin which had reacted at any time. The practical rate constants k_p as well as the corresponding k values are presented in Table 1.

In the case of the tertiary amine N:N-dimethylcysteamine the reaction was found to be nearly first order in any one run. The slight deviation from first order kinetics (Fig. 3) suggests that some intermediate product does accumulate. The most probable intermediate is S-penicilloyl-N:N-dimethylcysteamine, a compound which would be

TABLE 1. THE RATE OF INACTIVATION OF PENICILLIN BY VARIOUS MERCAPTOAMINES AND AMINES

Inactivator	pH	Conc. of reactants		k_p*	pK' of inactivator	k†
		Penicillin mM	Inactivator mM			
L(−)Cysteine	6·76	15	10	1·06	pK'_2 = 8·37	44·2
L(−)Cysteine	6·76	10	10	0·97	pK'_2 = 8·37	40·4
L(−)Cysteine	6·75	15	20	1·01	pK'_2 = 8·37	43·1
L(−)Cysteine	7·05	10	15	2·00	pK'_2 = 8·37	43·8
L(−)Cysteine	7·03	15	10	1·98	pK'_2 = 8·37	45·3
L(−)Cysteine	7·00	20	15	1·64	pK'_2 = 8·37	39·8
L(−)Cysteine	7·03	15	20	1·77	pK'_2 = 8·37	40·5
L(−)Cysteine	7·50	15	10	4·87	pK'_2 = 8·37	40·7
L(−)Cysteine	7·57	10	10	5·50	pK'_2 = 8·37	40·2
L(−)Cysteine	7·61	7·5	10	6·08	pK'_2 = 8·37	41·1
D(+)Cysteine	7·05	15	10	0·78	pK'_2 = 8·37	17·0
D(+)Cysteine	7·05	15	10	0·77	pK'_2 = 8·37	16·5
D(+)Cysteine	7·27	15	10	1·25	pK'_2 = 8·37	17·0
D(+)Cysteine	7·27‡	15	10	1·25	pK'_2 = 8·37	17·0
Cysteamine	7·07	10	7·5	1·02	pK'_2 = 8·35	20·5
Cysteamine	7·05	10	15	0·87	pK'_1 = 8·35	18·2
Cysteamine	7·05	20	15	0·98	pK'_1 = 8·35	20·6
Cysteamine	7·47	10	7·5	2·28	pK'_1 = 8·35	19·6
L(−)Cysteine ethyl ester	6·28	15	10	2·22	pK'_1 = 6·69	7·9
L(−)Cysteine ethyl ester	6·27	10	15	2·24	pK'_1 = 6·69	7·9
L(−)Cysteine ethyl ester	7·04	10	15	6·15	pK'_1 = 6·69	8·9
L(−)Cysteine ethyl ester	7·04	15	10	6·12	pK'_1 = 6·69	8·9
L(−)Cysteine ethyl ester	7·29	10	15	8·58	pK'_1 = 6·69	10·7
N:N-Dimethyl cysteamine§	7·05	10	10	0·41	pK'_1 = 7·70	2·2
N:N-Dimethyl cysteamine§	7·05	10	20	0·39	pK'_1 = 7·70	2·1
N:N-Dimethyl cysteamine§	7·06	20	10	0·39	pK'_1 = 7·70	2·1
N:N-Dimethyl cysteamine§	7·44	10	30	0·75	pK'_1 = 7·70	2·1
N:N-Dimethyl cysteamine§	7·49	10	10	0·82	pK'_1 = 7·70	2·2
Hydroxylamine	6·37	10	15	12·5	pK'_1 = 6·03	18·2
Hydroxylamine	7·05	10	7·5	18·0	pK'_1 = 6·03	19·7
Hydrazine	6·95	15	15	0·45	pK'_1 = 7·93 pK'_1 = 8·2	4·8 8·5
Hydrazine	6·95	20	15	0·42	pK'_1 = 7·93 pK'_1 = 8·2	4·4 7·8
Hydrazine	7·28	10	15	0·68	pK'_1 = 7·93 pK'_1 = 8·2	3·7 6·4
Hydrazine	7·31	20	15	0·66	pK'_1 = 7·93 pK'_1 = 8·2	3·4 5·8

* k_p (in l. × moles^{-1} × min^{-1}) is the practical rate constant calculated from equation 10.

† k (in l. × moles^{-1} × min^{-1}) is the rate constant for the ionized form of the inactivator (equation 9).

‡ 0·05 M NaCl added.

§ The rate constants are calculated from the initial rates observed.

The second order rate constants were determined at 23 °C in 0·5 M phosphate buffer. Ionic strength 1·37–1·40.

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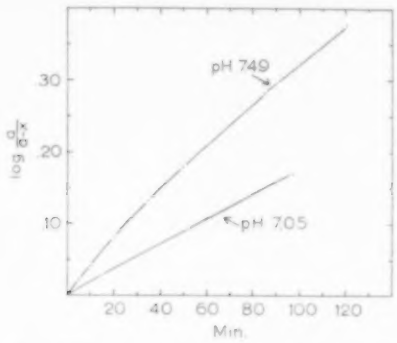


FIG. 3. First order rate plot of penicillin inactivation by N:N-dimethylcysteamine. Concentration of reactants 10^{-2} M. Temperature 23 °C.

expected to form hydroxamic acid with hydroxylamine. In experiments where the optical rotation and the amount of hydroxamic acid forming substance in solution were simultaneously measured (Fig. 4) evidence in favour of this hypothesis was obtained. It is clear, however, that the amount of intermediate formed is slight. It was found that the *initial* reaction rates were first order both with respect to penicillin and the inactivator. The second order rate constants thus determined, are presented in Table 1.

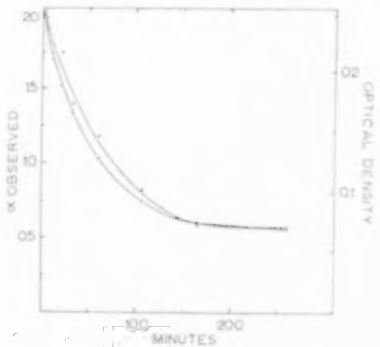


FIG. 4. The inactivation of penicillin by dimethylcysteamine. The concentration of penicillin was 10^{-2} M and that of dimethylcysteamine 3×10^{-2} M. pH 7·9, temperature 23 °C, M/15 phosphate buffer. Lower curve: The reaction followed by polarimetry. Upper curve: The hydroxamic acid formation measured spectro-photometrically in samples taken at different time intervals.

The effect of ionic strength

It can be seen from Table 1 that, whereas the practical rate constants k_p as expected increased considerably with increasing pH, the rate constants k (see equation 7) were, with one exception, independent of pH in the range studied. This finding demonstrates that the reactive form of the inactivator has released one proton. Thus, cysteamine and N:N-dimethylcysteamine will in their reactive form possess a zero net charge, whereas cysteine will have a net charge of minus one. Since penicillin possesses one

negative charge, an increase in the ionic strength would be expected to increase the reaction rate in the case of cysteine, but to have no effect in the case of cysteamine derivatives.[9] These expectations are borne out by the findings in Table 2. Although the Brönsted equation is not quantitatively obeyed because of the very high ionic strength in these experiments, the data seem to demonstrate that a bimolecular reaction (*i.e.*, the first step) is rate limiting.

TABLE 2. THE EFFECT OF SALTS ON THE RATE OF INACTIVATION OF PENICILLIN

Inactivator	pH	Salt added	k_p l mole^{-1} min^{-1}
L(−)Cysteine	6·96	None	1·94
L(−)Cysteine	6·96	3M NaCl	3·25
L(−)Cysteine	6·83	None	1·17*
L(−)Cysteine	6·83	3M Guanidine·HCl	2·20
D(+)Cysteine	6·84	None	0·48*
D(+)Cysteine	6·84	3M Guanidine·HCl	0·95
Cysteamine	6·93	None	0·73*
Cysteamine	6·93	3M Guanidine·HCl	0·74
N:N-Dimethyl-cysteamine	6·95	None	0·33*
N:N-Dimethyl-cysteamine	6·95	3M Guanidine·HCl	0·34

Concentration of penicillin 0·01 M, conc. of inactivator 0·015. The temperature was 23 °C. Otherwise conditions as in Table 1.

* Calculated on the basis of the data in Table 1.

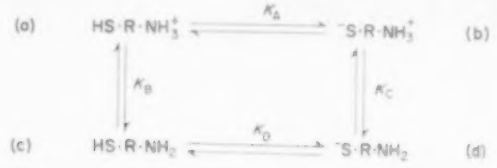
The effect of pH

In equation 7, pK is the titratable pK referring to the dissociation of one proton in the pH interval studied (*e.g.* the pK_1 in the case of cysteamine, and the pK_2 in the case of cysteine). It thus refers to the release of a proton from either the thiol or the amino group. If equation 9 is satisfied with a pK value which unequivocally refers to the thiol or the amino group, this finding constitutes evidence that the group in question is involved in the rate limiting step. For mathematical reasons, such an agreement will carry weight only when the experiments are performed in a pH interval close to the pK of the group in question. It appears from Table 1 that in most experiments this condition is fulfilled.

In the case of cysteamine, the dissociation constants of the thiol and the amino groups are so widely separated that in the pH range studied the pK_1 reflects almost exclusively the dissociation of the thiol group. The data of Table 1 therefore strongly indicate that the rate limiting step involves the ionized thiol. The same holds true for N:N-dimethylcysteamine, since its tertiary amino group undoubtedly is still more basic than the primary amine in cysteamine, and the pK_1 therefore refers unequivocally to the sulphydryl group.

The interpretation of some of the pH data of Table 1 is rendered difficult by the fact that a complicated relationship exists between the pK, as determined by titration, and the relative extent of dissociation of the two ionizable groups.

According to Benesch and Benesch[10] the mercaptoamines dissociate according to the following diagram:



It can be shown that the concentration of b relative to c is independent of pH. In the case of cysteine, cysteine ethyl ester, and cysteinylglycine, the dissociation constants for the sulphydryl and the amino group are so close that significant amounts of c will be present together with b. It can be calculated from the equilibrium constants given by Benesch and Benesch that for these compounds the ratio of b to c is 2·1, 0·21, and 0·19, respectively. In our experiments pH (6·3–7·3) the concentration of d is negligible for the mercaptoamines of Table 1, except for cysteine ethyl ester. For this compound appreciable amounts of the molecular species d will appear with increasing pH in the range studied. This fact may explain the finding (Table 1) that k rises with increasing pH, since the mercaptide group as well as the amino group of the molecular species d will be expected to possess a very high nucleophilic reactivity.

Since I^- of equation 6 equals b + c, the kinetic data fail to reveal which chemical group participates in the rate limiting step in the case of cysteine, cysteine ethyl ester and cysteinylglycine. Certain conclusions can, however, be drawn with the aid of data from other systems[11,12] (Table 3). The comparisons here carried out support the conclusion that the rate determining step involves the thiol group. Thus, it can be seen that when the rate constants are calculated on the basis of the concentration of the mercaptide

TABLE 3. THE RELATIVE RATES OF INTERACTION OF VARIOUS MERCAPTOAMINES AND AMINES WITH PENICILLIN, CYSTINE AND CYSTAMINE

Compound	pK	Penicillin			Cystine	Cystamine
		k	k_S^-	k_N	k_S^-	k_S^-
L(−)Cysteine	8·37*	42	63	126	25,000	19,500
Cysteamine	8·35*	20	20	>5000	12,500	9300
D(+)Cysteine	8·37*	17	25	53	—	—
L(−)Cysteinylglycine	7·07*	4–6	30–35	5–6	14,200	—
L(−)Cysteine ethyl ester	6·69*	8–12	50–90	11–18	9700	7800
N-Dimethylcysteamine	7·70†	2·1	2·1	2100	4600	—
N-Diethylcysteamine	7·80†	1·1	1·1	1100	5900	—
D(+)Penicillamine	7·90†	0·0004	—	—	4400	1400
AET	—	0·0	—	0	—	—
Hydroxylamine	6·03†	19	—	19	—	—
Hydrazine	~8†	4–8	—	4–8	—	—
D,L-Serine	9·15†	0·0	—	0·000	—	—
D,L-Serine methyl ester	7·05‡	0·0	—	0·000	—	—
Ethylenediamine	7·35	0·3	—	0·3	—	—

In the experiments with penicillin the k values are the same as those given in Table 1 whereas k_S^- and k_N are the corresponding rate constants obtained on the assumption that the ionized thiol group or the nucleophilic amine group, respectively, were responsible for the rate determining step. The rate constants k_S^- given in the reactions with cystine and cystamine refer to the interaction (at pH 7·4 and 37°C) of the ionized thiol with one of the sulphur atoms of the disulphides[11,12].

* Calculated from the data of Benesch and Benesch.[10]
† See Wintersteiner *et al.*[3]
‡ Own determination.

ions, the relative rate of interaction of cysteine, cysteamine, cysteinylglycine, N:N-dimethylcysteamine and N:N-diethylcysteamine with penicillin corresponds fairly well with their relative rates of reaction with disulphides. Cysteine ethyl ester, however, inactivates penicillin at a somewhat faster rate than could be expected from its nucleophilic reactivity observed in the other systems. As pointed out above, for this compound c/d = 0·21, *i.e.* the concentration of nucleophilic amine is far larger than that of the mercaptide ion. Although a direct aminolysis does occur in the case of hydroxylamine, hydrazine and ethylenediamine (Tables 1 and 3), such a mechanism is considered unlikely in the case of cysteine ethyl ester in view of the finding that serine methyl ester with a pK_N of 7·05 did not inactivate penicillin.

Altogether, the results from the pH-experiments strongly indicate that in the interaction of penicillin with mercaptoamines the rate limiting step involves the S^- ion.

Racemization

The final specific rotation in the case of the primary mercaptoamines (*i.e.* that of the penicilloylamides) falls in the same range as that of the free penicilloic acid formed upon incubation with penicillinase (Fig. 1). It is therefore likely that in the case of the primary mercaptoamines the opening of the β-lactam ring proceeds without racemization of any of the three optical carbon atoms.

Despite the fact that the present kinetic data confirm that the final reaction product in the case of the N:N-dialkylcysteamines is free penicilloic acid, it was found that the final specific rotation deviates considerably from that of the enzymatically prepared penicilloic acid. Apparently, in these cases the reaction mechanism involves a racemization of at least one of the three optical carbon atoms.

DISCUSSION

The data here presented seem to establish that a bimolecular reaction is rate limiting and, furthermore, that the rate limiting step involves the mercaptide ion. Since the optical measurements reflect the opening of the β-lactam ring, either the rate limiting step itself results in the opening of the ring, or it is effected by a fast subsequent step.

The fact that some amines react almost as rapidly as the aminothiols (Table 2) leads to an element of doubt in the proposed scheme. The data demonstrate that hydroxylamine and hydrazine react at a rapid rate, ethylenediamine and dimethylamine react at a moderate rate, whereas the amino groups of larger molecules such as serine and serine methyl ester are unable to react, in spite of the fact that the pK_N of serine methyl ester is 7·05. These findings indicate that the aminolysis is strongly influenced by steric factors, and it appears unlikely that the scission of the ring by mercaptoamines can be accounted for to any significant extent by a direct aminolysis.

A direct thiolysis is unlikely in view of the previously established facts (Wintersteiner *et al.*[3]) that a number of thiols and N-acetylated mercaptoamines are unable to inactive penicillin.

In our opinion the first step probably involves the formation of a semimercaptol, in accordance with equations 1–3. This view is supported by the fact pointed out by Woodward *et al.*[7] that the amide linkage of the strained β-lactam ring of penicillin is only to a limited extent stabilized by resonance, a circumstance which tends to confer ketonic properties on the amide carbonyl group.

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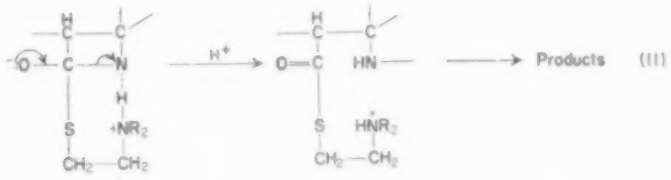
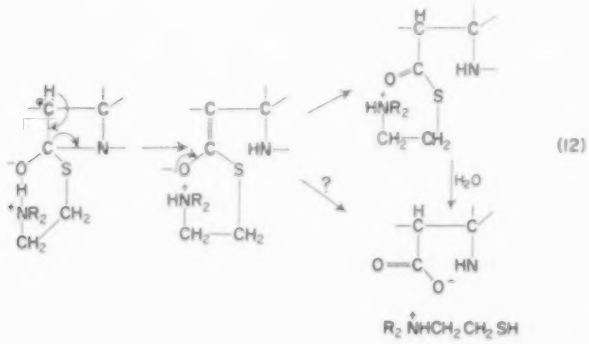
The breaking of the C–N bond of the β-lactam ring is obviously facilitated by the presence of the amino group. This effect can conceivably be brought about in several ways.

One possibility is that the amino group catalyzes the reaction by an electrostatic effect on the nitrogen atom as depicted in equation 11:



This possibility seems unlikely, however, as such a mechanism can not account for the fact that N:N-dimethylcysteamine, in contrast to the primary mercaptoamines, causes a reacemization of the penicillin. Also it is known that the tertiary nitrogen group of the β-lactam ring is not titratable, which renders it unlikely that this nitrogen atom can participate in hydrogen bond formation.

An alternative possibility is that the amino group of the mercaptoamine forms a hydrogen bond with the hydroxyl group of the semimercaptol. Such an interaction will be expected to facilitate the formation of a double bond (equation 12) with concomitant racemization of carbon atom no. 3. This mechanism might explain the observed racemization in the case of N:N-dimethylcysteamine. The product will presumably rearrange to the thioester which is subsequently hydrolyzed.



Thioesters in general are known to be fairly stable at pH 7. Recent data suggest, however, that an amine group in position β to the thiol strongly influences the rate of hydrolysis. Thus, Hansen[11] has found that 2-N:N-dimethylaminoethyl-thiolpropionate is hydrolyzed 240 times faster than the corresponding N-trimethyl derivative and that the Me_2HN^+ form reacts very much faster than the uncharged form of the amino group.

The small racemization, if any, which was observed in the case of primary mercaptoamines, can be satisfactorily explained by assuming reaction 4 to take place more rapidly than reaction 12. The initial semimercaptol formation will abolish the resonance

between a carbonyl group and a nitrogen atom and thereby lower the stability of the ring. Furthermore, a semimercaptol formation may be expected to lower the pK-value of the amine by way of an inductive effect along the carbon chain and thereby increase the concentration of the active nucleophilic amine. Obviously, the attack of the amino group is strongly favoured by its steric position.

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INVESTIGATIONS ON THE MODE OF ACTION OF *CYCLO*-SERINE UPON PROTEIN SYNTHESIS IN *ESCHERICHIA COLI*

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Abstract—D-*cyclo*serine and L-*cyclo*serine interactions with DL-alanine 1-C^{14} incorporation into the proteins of an *E. coli* strain were investigated.

The actions of the D- and L-isomers of *cyclo*serine on protein synthesis are closely related to alanine metabolism. The incorporation of algal protein hydrolysate C^{14} into bacterial proteins does not seem to be influenced by D- and L-*cyclo*serine.

A comparison of the effects promoted by D-*cyclo*serine and by D-alanine on the incorporation of DL-alanine-1-C^{14} into bacterial proteins shows that D-*cyclo*serine behaves like a D-alanine analogue.

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Previous investigations carried out in our laboratories[1] have shown a specific antagonism of D-α-alanine both on the inhibition of the bacterial growth and on protoplast formation by D-*cyclo*serine in *E. coli*.

In particular, these researches suggested that D-*cyclo*serine may interfere specifically with the bio ynthesis of a polypeptide-complex of the cell wall, containing D-alanine, considered necessary for the cell integrity.[2, 3]

The present paper deals with the action of *cyclo*serine upon protein biosynthesis by a mutant strain of *E. coli*.

In order to avoid complications resulting from parallel processes of growth, we utilized a thymine and phenyl-alanine requiring strain which synthesizes proteins under conditions of thymine starvation.

MATERIALS AND METHODS

(*a*) *Test organism. E. coli* 147, a thymine and phenylalanine requiring strain, was isolated in our laboratories.[4]

E. coli 147 protoplasts were obtained after ten hours incubation of a cell suspension in a medium containing 1250 units/ml of penicillin. We also used a cell-free supernatant prepared by centrifugation at 20,000 *g* of a protoplast suspension lysed by digitonin.[5]

(*b*) *Experimental media.* The synthetic medium of Davis, supplemented with Difco casein hydrolysate (25 μg/ml) and DL-phenylalanine (75 μg/ml), was used for cell suspensions of *E. coli* 147.

The synthetic medium of Davis, supplemented as above, and enriched with sucrose up to 20 per cent and $MgSO_4$ up to 0·2 per cent, was used for protoplast suspensions.

The density of the bacterial suspension in the incubation flasks was about 50–60 mg of cells (dry weight) per 100 ml of medium.

(*c*) *Incubation.* Suspensions were kept, without shaking, at 37 °C for 40 or 120 min according to experimental needs.

The bacterial cells were grown in a Davis medium containing limiting amounts of thymine, and were harvested when growth stopped from thymine exhaustion (48 hr). *E. coli* 147 cells are able to synthesize proteins even after thymine starvation, provided that phenylalanine is present.

(*d*) *Chemicals.* The labelled protein-precursors were an algal protein hydrolysate-C^{14} and DL-alanine-1-C^{14} supplied by the Radiochemical Centre of Amersham (England) while unlabelled natural amino-acids were from Ciba (Bale).

L-*cyclo*serine from synthesis (96 per cent pure) and D-*cyclo*serine from biosynthesis (98 per cent pure) were kindly supplied by the Farmitalia Chemical Laboratories of Settimo Torinese (Italy).

(*e*) *Protein extraction.* The protein metabolism of bacterial suspensions incubated with radioactive compounds was blocked by adding trichloracetic acid (TCA) to give a final concentration of 5 per cent. The protein fraction was purified by washing TCA-insoluble material three times with cold and twice with hot 5 per cent TCA (15 min at 95 °C), then once with ethanol and three times with hot ethanol–ether 3:1 (5 min at 65 °C).[6]

Finally the protein fraction was completely dried with ether.

(*f*) *Cell wall material.* Sometimes we used a residual cell fraction, previously extracted with hot TCA, resisting the tryptic digestion.[7] The specific activity of the residual fraction, probably containing some polypeptide compounds from the bacterial cell wall, was determined after extraction with hot ethanol–ether 3:1.

(*g*) *Radioactivity measurements.* In any case the radioactivity of the experimental samples was measured using 1 cm^2 or 2 cm^2 polyethylene planchets and thin mica-window Tracerlab G.M. tubes (TGC2) and employing, when necessary, self-absorption correction factors.

Duplicate samples were always prepared.

EXPERIMENTAL RESULTS

(*a*) *Action of* D-cyclo*serine on the incorporation of algal protein hydrolysate –C^{14} in bacterial proteins.* D-*cyclo*serine, in concentrations up to 40 μg/ml does not seem to influence the incorporation of algal protein hydrolysate-C^{14} in *E. coli* 147 proteins. On the other hand chloramphenicol, under the same experimental conditions, shows the known inhibiting action even at 25 μg/ml (Table 1).

TABLE 1. ACTION OF D-*cyclo*SERINE AND CHLORAMPHENICOL (CHL) ON THE INCORPORATION OF ALGAL PROTEIN HYDROLYSATE-C^{14} INTO PROTEINS OF *E. coli* 147

Experiment	Control	+ D-*cyclo*serine			+ CHL
		10 μg/ml	20 μg/ml	40 μg/ml	25 μg/ml
1	5,486	—	5,558	—	3,089
2	21,000	22,000	22,800	21,500	—

Values are given as cpm/mg of protein

Also in a cell-free system (supernatant at 20,000 × *g* of *E. coli* 147 protoplasts lysed by digitonin), D-*cyclo*serine up to 50 μg/ml does not cause variations in algal hydrolysate incorporation into proteins (Table 2).

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TABLE 2. ACTION OF D-*cyclo*SERINE ON THE INCORPORATION OF ALGAL PROTEIN HYDROLYSATE-C^{14} INTO PROTEINS OF AN *E. coli* CELL-FREE SYSTEM

Control	+ D-*cyclo*serine		
	12 μg/ml	25 μg/ml	50 μg/ml
574	551	545	553

Values are given as cpm/mg of protein

(*b*) *Action of* D-cyclo*serine on the incorporation of* DL-*alanine*-1-C^{14} *into bacterial proteins.* D-*cyclo*serine (20μg/ml) reduces remarkably DL-alanine-1-C^{14} incorporation into total proteins as well as into the cell wall fraction of *E. coli* 147, where the decrease is more evident (Table 3).

TABLE 3. ACTION OF D-*cyclo*SERINE ON THE INCORPORATION OF DL-ALANINE-1-C^{14} INTO PROTEINS AND CELL WALL FRACTION OF *E. coli* 147

Experiment No.	Total proteins		Cell-wall fraction	
	Control	D-*cyclo*serine (20 μg/ml)	Control	D-*cyclo*serine (20 μg/ml)
1	10,200	4,750	16,000	6,000
2	1,775	725	4,680	710

Values are given as cpm/mg of protein

However it is improbable that the primitive site of action of D-*cyclo*serine be situated in the bacterial cell wall; in fact a decrease in DL-alanine-1-C^{14} incorporation was demonstrated also in protoplasts (Table 4).

TABLE 4. ACTION OF D-*cyclo*SERINE ON THE INCORPORATION OF DL-ALANINE-1-C^{14} INTO PROTEINS OF PROTOPLASTS OF *E. coli* 147

Control	+ D-*cyclo*serine (25 μg/ml)
6,983	2,112

Values are given as cpm/mg of protein

However, DL-alanine-1-C^{14} incorporation into proteins does not decrease in proportion to the increase of antibiotic concentration. In particular insignificant variations in protein specific activity were observed when the antibiotic concentration ranged from 1·2 to 2·4 μM/ml. The data reported in Table 5 demonstrate the similar influence of equimolecular concentrations of unlabelled D-alanine, D-*cyclo*serine and unlabelled D-alanine + D-*cyclo*serine on the incorporation of DL-alanine-1-C^{14} into proteins.

Table 5. Effect of D-*cyclo*serine and unlabelled D-alanine on the incorporation of DL-alanine-1-C^{14} into proteins of *E. coli* 147

D-*cyclo*serine		D-alanine		D-*cyclo*serine + D-alanine	
μM/ml	cpm/mg	μM/ml	cpm/mg	μM/ml	cpm/mg
0	2,331	0	2,331	0	2,331
0·08	1,901	0·08	1,823	0·04 + 0·04	1,895
1·20	972	1·20	1,286	0·6 + 0·6	1,260
2·40	855	2·40	1,106	1·2 + 1·2	954

Values are given as cpm/mg of protein

(*c*) *Action of* L-cyclo*serine on the incorporation of algal protein hydrolysate-*C^{14} *and* DL-*alanine*-1-C^{14} *into proteins of E. coli* 147. L-*cyclo*serine at concentrations of 10·2 μg/ml does not cause variations in algal protein hydrolysate-C^{14} incorporation into proteins of *E. coli* 147 cells. On the other hand, at the same concentration, it considerably increases the total protein specific activity either in the whole cells or in protoplasts of *E. coli* 147, when DL-alanine-1-C^{14} is used as protein precursor (Table 6).

Table 6. Action of L-*cyclo*serine on protein synthesis of *E. coli* 147

Precursor	System	L-*cyclo*serine		
		0	10·2 μg/ml	25 μg/ml
Protein hydrolysate-C^{14}	Cells	7,622	7,297	—
DL-alanine-1-C^{14}	Cells	3,417	5,865	—
DL-alanine-1-C^{14}	Protoplasts	6,983	—	17,194

Values are given as cpm/mg of protein

(*d*) *Comparison of the effects of the enantiomorphs of* cyclo*serine and alanine on the incorporation of* DL-*alanine*-l-C^{14} *into the protein of E. coli* 147. To make clear the quantitative relationships of the observed antagonism between the enantiomorphs of *cyclo*serine and those of alanine, we compared the effects induced on DL-alanine-1-C^{14} incorporation into bacterial proteins by equimolecular amounts of D- and L-alanine and of D- and L-*cyclo*serine. DL-Alanine-1-C^{14} was used as a protein precursor and was added to the Davis medium enriched with 25 μg/ml of Difco casein hydrolysate and 75 μg/ml of DL-phenylalanine. The incubation time was generally reduced to 40 min.

The incorporation of a labelled precursor into a given compound is proportional, *ceteris paribus*, to the concentration of this precursor in its pool. In our case, any variation of DL-alanine specific activity in its pool, promoted by adding unlabelled L- or D-alanine as a diluent will occur according to the law of isotopic dilution and will be reflected by a corresponding variation of the protein specific activity.

Now if A is the activity of the amount b of a given amino acid in the pool, the specific activity is

$$a = \frac{A}{b}.$$

Adding a given amount x of the same amino acid unlabelled, the specific activity becomes

$$y = \frac{A}{b + x};$$

we can write the following proportion:

$$a : y = \frac{A}{b} : \frac{A}{b + x}$$

from which:

$$y = \frac{a}{1 + \frac{x}{b}} \tag{1}$$

Whenever the addition of an unlabelled amino acid induces no significant variations of protein synthesis or of the precursor pool size, the variations of protein specific activity, due to this addition, are expressed by equation (1), where y means the observed protein specific activity (as cpm/mg), x the amount of the diluent amino acid in the medium (as μM/ml), and a the protein specific activity of the control (made equal to 100).

Our experimental data concerning L-alanine dilution are in agreement with equation (1) (Fig. 1).

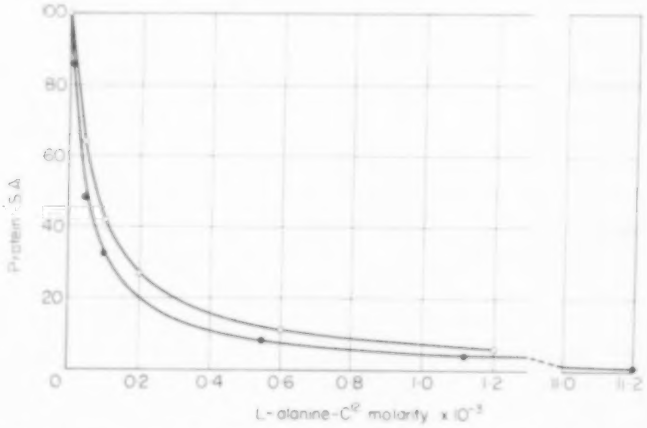

FIG. 1. Effect of exogenous L-alanine-C^{12} on the incorporation of DL-alanine-1-C^{14} into proteins Expt. No. 1 ○—○: 40 min incubation; $b = 0{\cdot}0747$. Expt. No. 2 ●—●: 120 min incubation; $b = 0{\cdot}0498$.

Considering $a = 100$, b can be determined according to:

$$b = x\left(\frac{y}{a - y}\right)$$

From Fig. 1 it is apparent that for high values of x, y tends to zero. Therefore it may be deduced that L-alanine is easily converted into its D-isomer, presumably without any variation in the L-alanine/D-alanine ratio in the pool of the precursors.

Molar concentrations of L-*cyclo*serine identical to those used for L-alanine did not induce similar effects (Figs. 1 and 2). The curve in Fig. 2 shows an increase in specific activity for the lowest doses of L-*cyclo*serine and a decrease for the highest ones.

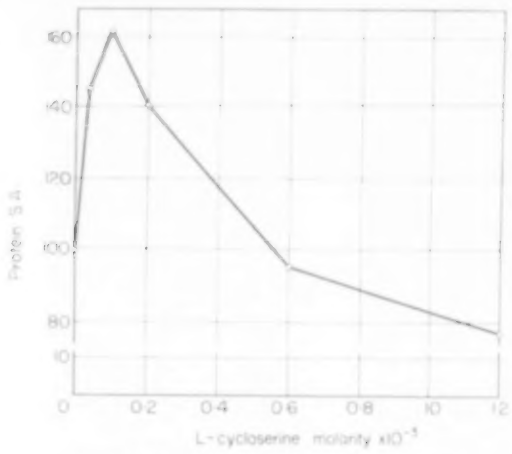

Fig. 2. Effect of L-*cyclo*serine on the incorporation of DL-alanine-1-C^{14} into proteins (40 min incubation).

When unlabelled D-alanine is added as diluent, the radioactivity incorporated into bacterial proteins from DL-alanine-1-C^{14} does not tend to zero as the amino acid concentration increases, but reaches a minimum value which thereafter remains constant. This value varies remarkably from one experiment to the other and ranges from 45 to 15 per cent of the activity incorporated into the control.

Supposing that under our conditions D-alanine is not converted into L-alanine, the minimum constant radioactivity which appears in proteins in presence of high levels of unlabelled D-alanine should represent only the incorporation of L-alanine-1-C^{14}.

Therefore the dilution process should be represented by:

$$y = C + \frac{a - C}{1 + \frac{x}{b}} \tag{2}$$

where C is the minimum constant value of the protein specific activity, due to the presence of L-alanine-1-C^{14}.

Considering $a = 100$, b can be determined according to:

$$b = x \frac{y - C}{a - y}$$

D-alanine dilution curves appear to fit quite well with equation (2), where y tends to C when x tends to high values (Fig. 3).

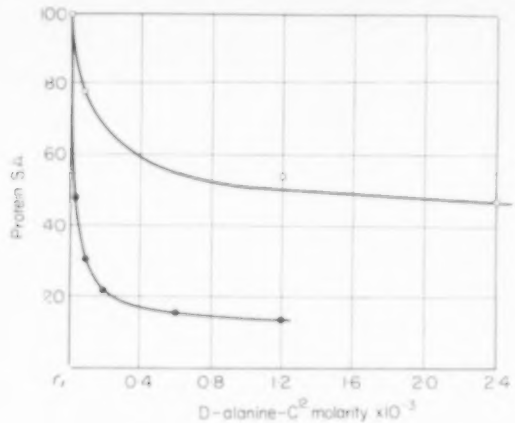

FIG. 3. Effect of exogenous D-alanine-C^{14} on the incorporation of DL-alanine-1-C^{14} into proteins. Expt. No. 1 ○—○: 40 min incubation; $b = 0{\cdot}14$, $c = 45$. Expt. No. 2 ●—●: 40 min incubation; $b = 0{\cdot}023$, $c = 13$.

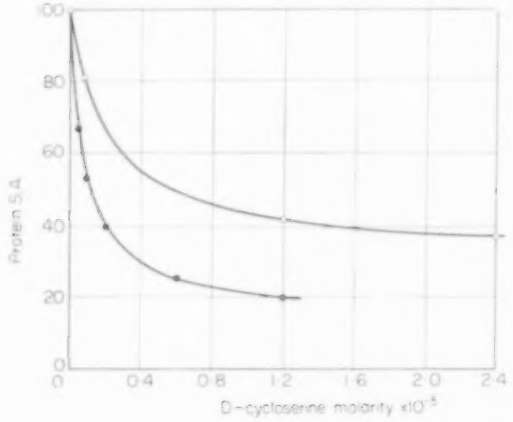

FIG 4. Effect of D-cycloserine on the incorporation of DL-alanine-1-C^{14} into proteins. Expt. No. 1 ○—○: 40 min incubation; $b = 0{\cdot}20$, $c = 32$. Expt. No. 2 ●—●: 40 min incubation; $b = 0{\cdot}08$ $c = 15$.

The curves represented by the same symbol belong to the same experiment.

Also the curves of protein specific activity plotted against molar concentrations of D-*cyclo*serine, in the same range as those used for D-alanine (Fig. 3 and Fig. 4), appear to be in agreement with equation (2).

The effects promoted by L- and D-alanine and D-*cyclo*serine on the incorporation of DL-alanine-1-C^{14} in the cell wall fraction were also investigated. The results were in agreement with those obtained for incorporation into total proteins. In fact, the decrease of the specific activity promoted by D-alanine and D-*cyclo*serine was even more evident in the cell wall fraction than in total proteins (Table 7).

Table 7. Effect of L-alanine, D-alanine and D-*cyclo*serine on the DL-alanine-1-C^{14} incorporation into total proteins (TP) and cell wall fraction (CWF) of *E. coli* 147

Experiment No.	Control		L-alanine (9 μg/ml)		D-alanine (9 μg/ml)		D-*cyclo*serine (25 μg/ml)		D-*cyclo*serine (25 μg/ml) L-alanine (9 μg/ml)		D-*cyclo*serine (25 μg/ml) D-alanine (9 μg/ml)	
	TP	CWF	TP	CWF	TP	CWF	TP	CWF	TP	CWF	TP	CWF
1	3924	—	1387	—	1832	—	1186	—	700	—	1300	—
2	711	1300	—	—	—	—	382	500	248	450	335	480
3	1738	2220	1052	1372	1542	1492	994	963	632	678	960	893

DISCUSSION

Our results showed that the action of D-*cyclo*serine is closely related to the utilisation of D-alanine in the protein biosynthesis of *cyclo*serine-sensitive bacterial cells and protoplasts.

Buogo *et al.*[1] made evident a competitive antagonism of D-*cyclo*serine with D-alanine which can be quantitatively studied by analysing the DL-alanine-1-C^{14} incorporation into bacterial proteins in the presence of variable amounts of unlabelled D- or L-alanine and D- or L-*cyclo*serine. By addition of increasing amounts of unlabelled L-alanine, the radioactivity incorporation into proteins tends to zero as if the amino acid could dilute both the L- and D- isomers of the labelled amino acid racemate. This is in agreement with the hypothesis of the enzymatic conversion of L-alanine into D-alanine.

On the contrary, the DL-alanine-1-C^{14} incorporation decreases after addition of unlabelled D-alanine but instead of tending to zero, reaches a minimum constant value which is probably accounted for by the fact that the L-fraction of the alanine-1-C^{14} racemate does not undergo any dilution effect and therefore it should be concluded that there is no apparent conversion of D-alanine into L-alanine under our experimental conditions, in spite of our knowledge on the behaviour of the bacterial alanine-racaemase[8].

The decrease of DL-alanine-l-C^{14} incorporation into proteins caused by increasing amounts of unlabelled D-*cyclo*serine is quantitatively similar to that observed after addition of unlabelled D-alanine, and here again the radioactivity does not decrease below a given minimum value when further amounts of this antibiotic are added. It has been observed that at the lowest concentrations used in our experiments D-alanine and D-*cyclo*serine showed additional effects (Table 5).

Buogo *et al.*,[1] observing that D-*cyclo*serine acts on sensitive micro-organisms requiring thymine and phenylalanine, provided that phenylalanine be present, that is, when conditions for protein biosynthesis obtain, were led to the conclusion that the D-*cyclo*serine action is closely related to the protein synthesis.

The observations described in the present paper show that this action is limited to the part played by alanine in protein synthesis.

Our results support the hypothesis that D-*cyclo*serine behaves as a D-alanine analogue during bacterial protein synthesis. It, or one of its derivatives, might replace the natural amino acid in protein formation.

The inhibiting action of D-*cyclo*serine on the protein incorporation of DL-alanine-l-C^{14} is more evident in the cell wall fraction than in total cellular protein.

As a significant decrease in DL-alanine-1-C^{14} incorporation is observed also in the protein of intact protoplasts, it is likely that the site of action of *cyclo*serine is located in the protoplasm, where the synthesis of the peptide-complex of the wall, containing D-alanine, may occur.

The observation that the D-*cyclo*serine inhibiting action appears to be greater on the synthesis of cell wall constituents may be explained by the larger amounts of D-alanine occurring in the cell wall than in the intracellular proteins.

The action of the L-isomer of *cyclo*serine appears to be quite different from that of D-isomer. In our experiments, L-*cyclo*serine at low concentrations enhanced DL-alanine-1-C^{14} incorporation into both bacterial cells and protoplast proteins. Only higher concentrations induced inhibiting effects.

In any case we cannot exclude that the effects shown by L-*cyclo*serine could be due to some traces of impurities present in the batch of this compound which we used.

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STUDIES IN THE MOUSE OF THE PHARMACOLOGY OF 5-IODODEOXYURIDINE, AN ANALOGUE OF THYMIDINE

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Abstract—5-Iododeoxyuridine labeled with I^{131} or tritium was administered to normal and tumor-bearing mice, and a study was made of its metabolism, tissue distribution and excretion. The main pathway for degradation of IUdR involved a cleavage to 5-iodouracil and subsequent dehalogenation with the formation of uracil and inorganic iodide. Considerable iodination of non-nucleic acid derivatives was observed in the protein fractions of cells. The occurrence of iodinated derivatives of acyclic products of iodouracil catabolism was not demonstrated, although these probably were formed as a result of direct degradation. The acute LD_{50} of IUdR in mice was 2·5 g/kg. The LD_{50} for IUdR upon chronic administration intraperitoneally for 13 days was 318 mg/kg; this toxicity could be prevented completely by the prior administration of thymidine.

5-IODODEOXYURIDINE (IUdR),[1] an analogue of thymidine, is an effective inhibitor of the growth of several neoplasms in mice[2] and a competitive antagonist of the utilization of thymidine in microbial[1, 3] and mammalian[1, 2, 4, 5] systems. Studies of the mechanism of action of the analogue in mouse Ehrlich ascites tumor cells[1, 6] disclosed an inhibition of the utilization of precursors of pyrimidines for the biosynthesis of DNA-thymine, but not for RNA-uracil and -cytosine or DNA-cytosine. Because IUdR was utilized, in competition with thymidine, for which it substituted in the biosynthesis of DNA[4, 5, 7, 8, 9] and since it inhibited the incorporation of thymidylic acid into DNA in a cell-free system;[10] it is suggested that the site of inhibition is at the mono- or tri-phosphate level.

The present report is a study of the metabolism, tissue distribution, excretion and toxicity of IUdR in the mouse.

MATERIALS AND METHODS

Preparation of compounds. IUdR was synthesized by the procedure described previously[1], and I^{131}-labeled material was prepared by a modification of the method used for the synthesis of 5-iodouridine-I^{131}.[11] Tritiated IUdR was prepared from deoxyuridine labeled in position-5 and -6 with tritium.

Preparation and analysis of tissues. IUdR-I^{131} was administered intraperitoneally to normal mice and to mice* bearing the L-5178-Y lymphoma.† The tissues were removed at various intervals of time; these were either dissolved in hot 6N NaOH and adjusted to a constant volume or were fractionated by the Schneider modification[12] of the Schmidt–Thannhauser procedure[13] into cold acid-soluble, combined nucleic acids,

* Mice bearing L-5178-Y were AKR × DBA/2 F_1 hybrids, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

† The Y-strain of leukemia L-5178 was developed by G. A. Fischer and J. J. Jaffe from a single cell, isolated *in vitro* and grown initially in a medium which has been described by G. A. Fischer and A. D. Welch, Science, **126**, 1018 (1957). The original donor mice carrying L-5178 were kindly provided by L. W. Law of the National Cancer Institute.

and protein fractions. The protein fraction was dissolved in warm 6N NaOH and each of the three fractions from each tissue was brought to a common volume. The concentration of radioactive iodine was determined using a well-type gamma scintillation counter and the concentration of tritium either by using a liquid phosphor counter or by plating aliquots on stainless steel planchets and counting in a windowless gas-flow Geiger counter.

RESULTS AND DISCUSSION

Organ distribution

The amount of radioactivity in a specific tissue and the relative distribution varied considerably between different experiments and may be a result of different dosage

TABLE 1. TISSUE DISTRIBUTION OF RADIOACTIVITY IN NORMAL AND TUMOR-BEARING MICE INJECTED INTRAPERITONEALLY WITH A SINGLE DOSE OF RADIOACTIVE SODIUM IODIDE OR 5-IODODEOXYURIDINE*

Organ	Iododeoxyuridine				Sodium Iodide	
	0·5 hr		21 hr		21 hr	
	Tumor-bearing	Control	Tumor-bearing	Control	Tumor-bearing	Control
Tumor	21,300	—	3,650	—	200	—
Kidney	49,300	38,400	397	200	183	89
Spleen	26,000	26,600	4,480	3,180	25	32
Lymph node	20,600	23,000	3,400	2,000	13	290
Intestine	30,800	21,400	4,000	2,950	120	35
Liver	29,800	27,500	350	174	116	56
Heart	19,200	17,400	295	225	None	17
Lung	21,000	25,400	824	355	114	20
Thyroid	17,100	17,200	1,000	1,000	2,800	1,630
Testes	15,000	14,400	735	740	130	41
Brain	2,600	2,700	None	50	None	None
Thymus	16,100	19,600	3,420	4,400	320	105
Stomach	49,200	42,600	2,180	1,860	504	350
Skin	15,600	21,000	1,800	720	1,050	220
Muscle	12,200	13,900	311	145	130	167
Bone marrow†	2,600	2,900	1,400	776	50	32

* Both NaI and IUdR were labeled with I^{131}. Two groups of three mice each received intraperitoneally a single injection of 5 μmoles $4{\cdot}0 \times 10^6$ counts/min per μmole of the indicated compound. After the appropriate time the tissues were removed, weighed and dissolved or suspended in KOH (6 N; to 10 ml). The concentration of radioactivity is given in counts/min per gram of wet weight of tissue.

† Counts/min per tissue, rather than counts/min per g.

regimens employed in the administration of radioactive IUdR-I^{131}, as well as variations in the time of sacrifice of the mice. In Table 1 is shown the tissue distribution of radioactivity observed when tumor and non-tumor bearing mice* received a single injection of NaI^{131} (5 μmoles) or IUdR-I^{131} (5 μmoles) and were sacrificed after 0·5 or 21 hr. There was no striking difference between the tissues of normal mice and those bearing tumors in the localization of radioactivity derived from either compound and such differences which were observed may be insignificant because of the relatively small

* Mice used in studies of the toxicity and tissue distribution of IUdR-I^{131} were Swiss females, obtained from Millerton Research Farm, Millerton, N.Y.

number of animals used. The concentration of radioactivity in the thyroid tissue was 14-fold in excess of the amount present in tumor tissue in those animals injected with NaI^{131}, whereas in mice treated with IUdR-I^{131} this relationship between the tissues was reversed; thus, the tumor had a 3-fold excess of radioactivity, as compared with that of the thyroid glands. The latter tissue had an appreciable concentration of radioactivity, a finding which indicated that a considerable amount of breakdown of IUdR to iodide occurred; this catabolic degradation was confirmed subsequently by experiment.

In Table 2 is shown the distribution observed when two groups of mice each bearing the L-5178-Y lymphoma received intraperitoneal injections of IUdR-I^{131} (10 μmoles) and were sacrificed 2 and 24 hr later, respectively. Consideration of the data of Tables 1, 2 and 3 indicates that there was a rapid drop in the tissue concentration of radioactivity within 2 hr of the time of administration of IUdR, and that this decrease was followed by a relatively slow rate of disappearance of radioactivity during the succeeding 48 hr. There was considerable retention of radioactivity even after 24 hr and the decrease in concentration of radioactivity in the tumor at 24 hr following treatment was only to about 50 per cent of the amount present 2 hr following the administration of the analogue (Table 2).

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Table 2. Distribution of radioactivity in the acid-soluble, nucleic acid and protein fractions of tissues from mice bearing the 5178-Y lymphoma following injection of a single dose of radioactive 5-iododeoxyuridine*

Time of Sacrifice	Tissue	Wet wt. (g)	Concentration of radioactivity†		
			Acid-soluble	Nucleic acid	Protein
2 hr	Tumor	0·61	600	4,200	4,200
	Liver	3·80	100	560	314
	Spleen	0·34	3,300	2,500	3,900
	Kidney	1·07	40	2,550	980
	Stomach	0·59	1,600	430	300
	Intestine	8·30	730	73	35
	Heart	0·56	427	400	197
	Bone marrow‡	—	74	680	970
24 hr	Tumor	0·83	None	1,520	1,860
	Liver	4·04	30	380	218
	Spleen	0·50	710	1,000	2,200
	Kidney	1·13	20	650	440
	Stomach	0·52	55	173	173
	Intestine	7·68	80	60	37
	Heart	0·50	100	400	290
	Bone marrow‡	—	60	70	440

* Six mice were injected intraperitoneally with radioactive iododeoxyuridine labeled with I^{131} (10 μmoles; $6{\cdot}1 \times 10^6$ counts/min per μmole). Groups of three mice were sacrificed 2 and 24 hr later by severing the spinal cord and the tissues were removed rapidly, weighed and frozen in a bath of alcohol and dry-ice. The combined organs from each group of mice were homogenized in ice-cold trichloroacetic acid (TCA) (5 per cent; 25 ml) for 3 min. The residue after centrifugation was extracted two additional times with cold TCA, prior to extraction with hot TCA (5 per cent; 25 ml; 90°) for 30 min. The residue, composed primarily of protein, was dissolved in warm alkali.

† Values are expressed in counts/min per g of wet wt. of tissue.

‡ Tissue included bone and the values given are for the total content of radioactivity in counts/min.

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN THE ACID-SOLUBLE, NUCLEIC ACID AND PROTEIN FRACTIONS OF TISSUES FROM MICE BEARING THE 5178-Y LYMPHOMA FOLLOWING THE INJECTION OF RADIOACTIVE SODIUM IODIDE OR 5-IODODEOXYURIDINE*

Compound injected	Tissue	Wet wt. (g)	Concentration of radioactivity†			
			24 hr			
			Acid-soluble	Nucleic acid	Protein	Nucleic acid: Protein
Sodium Iodide-I^{131}	Tumor	1·67	None	None	12	0·0
	Liver	4·00	None	10	76	0·13
	Spleen	0·55	None	None	88	0·0
	Kidney	0·94	None	29	188	0·15
	Stomach	1·10	146	18	99	0·18
	Intestine	8·9	None	9	83	0·11
	Lung	0·58	None	None	59	0·0
	Thyroid	0·46	None	None	16	0·0
	Heart	0·43	None	None	None	—
Iododeoxy-uridine-I^{131} plus NaI	Tumor	0·90	None	391	300	1·3
	Liver	4·40	None	30	59	0·5
	Spleen	0·45	None	460	292	1·6
	Kidney	0·91	None	40	22	1·8
	Stomach	0·67	None	204	163	1·3
	Intestine	8·62	None	142	342	0·4
	Lung	0·55	None	285	157	1·8
	Thyroid	0·47	None	161	84	1·9
	Heart	0·34	None	None	None	—
				48 hr		
Sodium Iodide-I^{131}	Tumor	2·11	None	None	None	—
	Liver	4·61	None	None	32	—
	Spleen	0·54	None	None	None	—
	Kidney	0·95	None	None	56	—
	Stomach	0·49	None	None	None	—
	Intestine	8·5	None	None	10	—
	Lung	0·52	None	None	None	—
	Thyroid	0·41	None	None	116	—
	Heart	0·44	None	None	70	—
Iododeoxy-uridine-I^{131} plus NaI	Tumor	1·62	None	87	62	1·4
	Liver	4·10	None	17	16	1·0
	Spleen	0·42	None	170	130	1·3
	Kidney	0·93	None	24	59	0·4
	Stomach	0·57	None	53	96	0·6
	Intestine	7·50	None	9	27	0·3
	Lung	0·47	None	55	49	1·1
	Thyroid	0·55	None	None	40	—
	Heart	0·51	None	None	39	—

* Six mice were injected intraperitoneally 4 times during a 3-day period with NaI^{131} ($1·5 \times 10^5$ counts/min) and another group of 6 mice with $IUdR\text{-}I^{131}$ (5μmoles; $1·2 \times 10^6$ counts/min) plus non-radioactive sodium iodide (5 mg). Twenty-four and 48 hr following the last injection the animals were sacrificed and the tissues analyzed in the manner described in Table 2.

† Values are expressed in counts/min per g of wet wt. of tissue.

Fractionation of tissues

The tissues of mice which had received $IUdR\text{-}I^{131}$ were fractionated into cold acid-soluble, combined nucleic acids, and protein fractions, and their content of radioactivity was determined (Table 2). The radioactivity in the acid-soluble fraction derived

from the tumor tissues completely disappeared within 24 hr, while that of other tissues showed a marked decrease. The lowering of the concentration of radioactivity in the protein and nucleic acid fractions ranged from 25–75 per cent.

Although incorporation of a pyrimidine into DNA has been demonstrated by many investigators to lead to a relatively stable substance, i.e., with but little "turn-over", Zamenhof *et al*[14] have observed that halogenated pyrimidines may undergo an exchange reaction in the DNA of micro-organisms. Whether the observed decrease in the radioactivity of the nucleic acid fraction was a reflection of such a phenomenon (exchange with thymine) or of the loss of non-nucleic acid components associated with this fraction, either physiologically or as a result of the method of fractionation employed, is unknown and should be investigated. It is known that hot trichloroacetic acid (TCA) does produce partial hydrolysis of proteins and hence the "nucleic acid" fraction does contain amino acids, of which some may contain radioactive iodine. However, it has been shown that IUdR-I^{131} is incorporated into DNA[4, 5, 7, 8, 9] but what percentage of the observed radioactivity in this fraction represented *bona fide* radioactive nucleic acid has not yet been determined.

The following study was conducted in an attempt to minimize non-specific absorption of radioactive iodide, as well as the formation of non-nucleic acid reaction products such as iodinated amino acids. Non-radioactive sodium iodide (5 mg) was administered together with IUdR-I^{131} (5 μmoles) to two groups of mice which were sacrificed 24 or 48 hr after the last of 4 injections. Two additional groups received NaI^{131} alone and were sacrificed at the same intervals of time. The results are shown in Table 3. The radioactivity in the NaI^{131}-treated animals was incorporated primarily into the protein fraction and the amount of radioactivity present in the nucleic acid fraction disappeared completely within 48 hr. Whereas the administration of NaI^{131} alone resulted (after 24 hr) in a ratio of the radioactivity in the nucleic acid to protein fraction of less than 0·2, the concentration of radioactivity in the animals treated with IUdR-I^{131} was generally in favor of the nucleic acid fraction. In agreement with the previous experiment (Table 2), there was a marked decrease in the content of radioactivity in the nucleic acid fraction.

Metabolic derivatives of IUdR

To obtain information concerning the metabolism of IUdR the cold acid-soluble fraction of various tissues, as well as the urinary excretion products from mice with radioactive IUdR, were studied.

Twenty-eight young adult (22–25 g) Swiss female mice bearing a heavy population of Ehrlich ascites tumor cells (each mouse received about 5×10^7 cells intraperitoneally 4 days previously) were injected with 12 μmoles of IUdR-I^{131} divided into 4 doses given at 12 hr intervals. Three hours following the last injection the animals were sacrificed and the ascites tumor cells were collected by centrifugation with a total yield of 24·7 g wet-weight. The cells were extracted with ice-cold $HClO_4$ (0·5N; 250 ml) and following neutralization with KOH the formed $KClO_4$ was removed by filtration. The filtrate was adjusted to pH 9·8 and passed through a Dowex-1 formate column (2·5 cm $\times$ 30 cm). The ion exchange chromatogram obtained by gradient elution is shown in Fig. 1. Two radioactive areas, A and B, were obtained. Fraction A was mixed with non-radioactive iodouracil and iododeoxyuridine and aliquots were chromatographed on paper using the ethyl acetate–phosphate buffer system,[15] as well

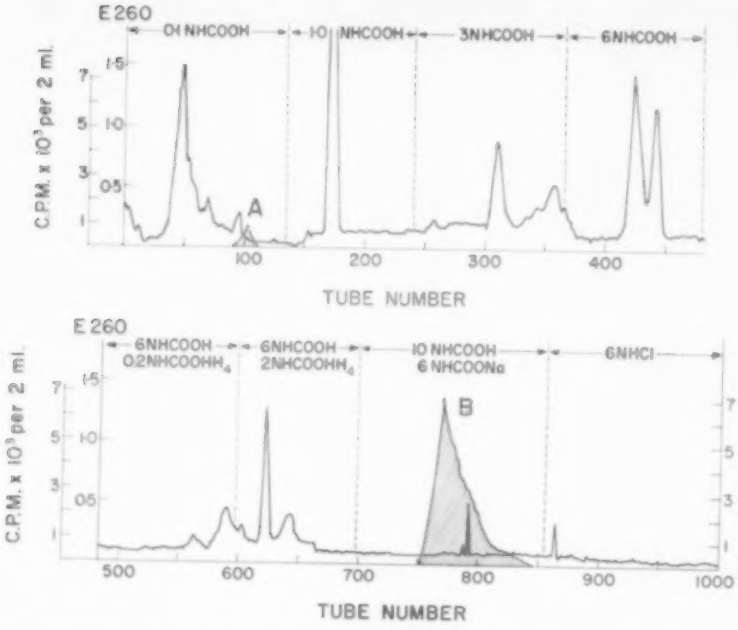

FIG. 1. Elution pattern of metabolic derivatives of IUdR-I^{131} from the cold acid-soluble fraction of Ehrlich ascites tumor cells (see text for details). Shaded area indicates radioactivity.

as on a Dowex-1 formate column (Fig. 2). Under both conditions the radioactivity coincided with that of iodouracil. Fraction B contained more than 95 per cent of the radioactivity in the acid-soluble fraction and was identified as iodide ion (see below).

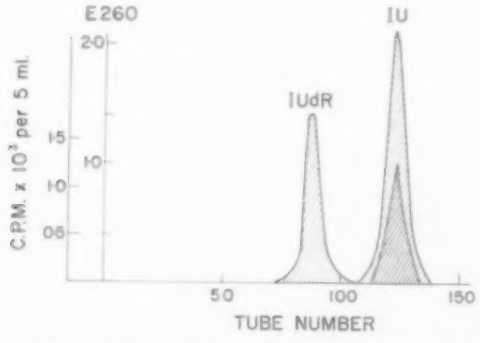

FIG. 2. Identification of fraction A (Fig. 1). Radioactive fraction A was mixed with non-radioactive 5-iodouracil and 5-iododeoxyuridine adsorbed on a Dowex-1 formate column (30 cm × 2·5 cm) and eluted by gradient elution with 0·1N HCOOH according to the method of Hurlbert *et al.*[22] Each tube contained 5 ml of effluent (see text for details). Cross lined area indicates radioactivity.

Rate of excretion of IUdR

The rate of excretion of radioactivity following the intraperitoneal injection of a single dose of IUdR-I^{131} is shown in Table 4. Within 4 hr approximately 74 per cent

of the radioactivity administered had appeared in the urine, and maximum excretion (91 per cent) was observed within 24 hr.

Urinary metabolic derivatives of IUdR

Each of four mice was injected with IUdR-I^{131} (5 μmoles; $4{\cdot}2 \times 10^6$ counts/min; 1.0 ml) and the urine was collected in the manner described in the footnotes of Table 4.

Table 4. Cumulative excretion of radioactivity in the urine following the intraperitoneal administration of IUdR-^{131} to mice*

Time (hr)	Total radioactivity (counts/min $\times 10^5$)	Per cent of administered dose excreted†
4	6·1 6·4 6·2	73·8
8	6·5 6·4 6·8	77·8
12	6·8 6·3 8·1	83·9
24	7·9 7·4 7·6	90·5
48	8·2 7·8 7·0	90·8

* Fifteen mice were placed individually in glass beakers and the urine was collected on filter paper protected by a wire screen. Each mouse received a single injection of IUdR-I^{131} (5 μmoles; $8{\cdot}4 \times 10^5$ counts/min; 1·0 ml) and 3 animals were sacrificed at each of the indicated times. The radioactivity was eluted from the filter paper with water in a Waring blendor, filtered, and an aliquot counted in a gamma scintillometer.

† Average for the group of 3 mice.

The pH of the urine collected during 24 hr was adjusted to 10·5, passed through a Dowex-1 formate column (200–400 mesh; 30 cm $\times$ 2·5 cm) and the latter was subjected to gradient elution with formic acid (2N), followed by formic acid (6N), NaCl (6N), and KI (6N). Three major radioactive peaks were obtained (Table 5). Iododeoxyuridine and iodouracil were eluted by low acid concentration; however, the free iodide appeared in the eluate only after KI was passed through the column. Following the administration of IUdR-I^{131}, radioactive iodide was the major radioactive substance present in the urine; in fact, it accounted for 83 per cent of the radioactivity present in the urine. Identification of this radioactive substance as iodide was made by the addition of concentrated HNO_3 (1 ml) to a 5 ml aliquot of the eluate, followed by continuous extraction with ether. Another aliquot was treated similarly with nitric acid, but the extraction was done with $CHCl_3$. The amount of radioactivity which appeared in the combined organic phases accounted for 97 per cent of the radioactivity present originally. This evidence, in addition to the failure of 6N NaCl, but the successful use of 6N KI, in the elution of the radioactive material from the Dowex-1 column, was

interpreted as identification of free iodide. The major non-radioactive substance was identified as uracil on the basis of its UV-spectra in acid and alkali, as well as its mobility on paper using several solvent systems. Furthermore, experiments to be described below with IUdR-6-H^3, unequivocally prove that the uracil was a metabolic derivative of IUdR.

TABLE 5. EXCRETION OF SOME METABOLIC DERIVATIVES OF 5-IODODEOXYURIDINE IN THE URINE OF MICE FOLLOWING THE ADMINISTRATION OF A SINGLE DOSE OF 5-IODODEOXYURIDINE-I^{131}*

Radioactive metabolic product	Per cent in urine
Iododeoxyuridine	6·6
Iodouracil	3·8
Iodide	83·2

* Each mouse was injected with 5 μmoles of IUdR-I^{131} and the urine was collected and measured as described in Table 4.

The following experiment was performed because the urine in the above experiment had been collected on filter paper with the concomitant possibility that the formation of iodide may have originated as a result of bacterial degradation following excretion, rather than being a *bona fide* metabolic derivative. Hence, when mice bearing the Ehrlich ascites tumor cells were injected with IUdR-I^{131} (see above and Fig. 1), the urine was collected in a tube suspended in a dry ice–alcohol bath. The pH of the urine was adjusted to 10·5, filtered, adsorbed on a Dowex-1 formate column ($\times$ 8; 200–400 mesh; 30 cm $\times$ 2·5 cm) and subjected to gradient elution (Fig. 3). A minimum of 7 radioactive peaks was observed; of these 7, 3 were identified as IUdR, iodouracil and iodide, respectively. The first two radioactive areas were re-chromatographed separately on Dowex-1 formate columns and the UV-spectra in acid and alkali were identical to those of IUdR[1] and iodouracil, respectively.

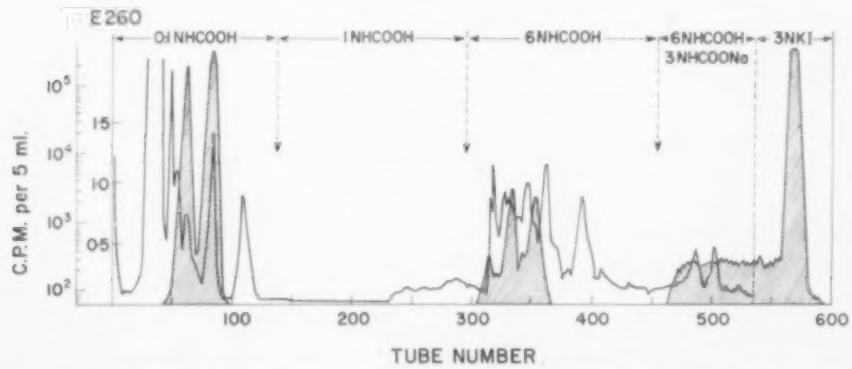

FIG 3. Elution pattern of urine from mice injected with IUdR-I^{131} (see text for details). Shaded area indicates radioactivity.

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Metabolism of tritiated IUdR

Because large amounts of free iodide formed following the administration of IUdR-I^{131} under *in vivo* and *in vitro* conditions, it was of interest to identify the deiodinated reaction products. Accordingly, 10 mice were injected with IUdR-6-H^3 (3·16 μmoles; 3 μc) and the urine was collected in a tube suspended in an alcohol–dry ice bath. The urine was adjusted to pH 10·8 with NH_4OH, absorbed on a Dowex-1 formate column (30 cm × 2·5 cm), and subjected to gradient elution (Fig. 4); seven radioactive areas were observed. The first radioactive peak corresponded to the major UV-absorbing area and this area following reduction in volume was subjected to paper chromatography in three solvent systems (*iso*propanol-HCl,[16] butanol–H_2O,[17] and ethyl acetate–phosphate buffer (pH 6·0[15]). Two radioactive and two Uv-absorbing areas were identified, and in all three solvent systems one of the Uv-absorbing areas possessed radioactivity. Neither area contained a deoxyribose group, as evidenced by a negative reaction for this sugar using the method of Stumpf.[18] The Uv-area which was radioactive had the spectral properties characteristic of uracil, and, in the three paper chromatograms, this material had an R_F value which was identical to that of uracil. The other radioactive area, which contained the larger proportion of radioactivity, did not coincide with the other Uv-absorbing area. On the basis of paper chromatography in several solvent systems this major radioactive area was not dihydrouracil, ureidopropionic acid or β-alanine. The specific activity of the uracil isolated was 30,800 counts/min per μmole and hence represented a 32-fold dilution of the specific activity of the administered IUdR-H^3. Whether iododeoxyuridine resulted in an inhibition of the degradation of uracil, in a manner comparable to that reported for azathymine,[19] should be investigated.

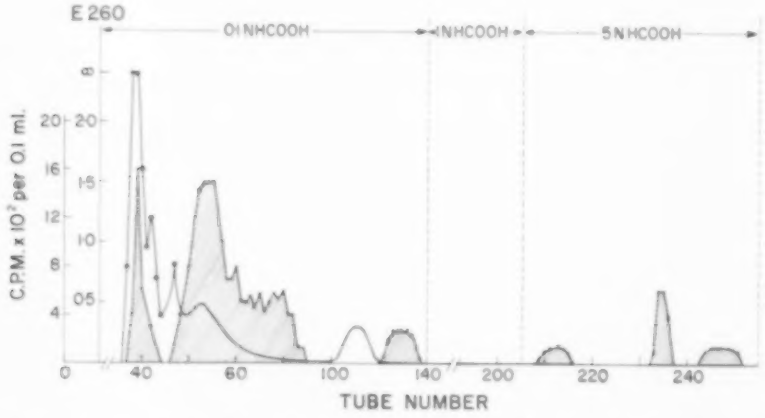

Fig. 4. Elution pattern of urine from mice injected with IUdR-H^3 (see text for details). Shaded area indicates radioactivity.

Toxicity of iododeoxyuridine

Acute toxicity from IUdR in Swiss mice resulted from the intraperitoneal administration of quantities in excess of 1·25 g/kg (Table 6); the LD_{50} was approximately 2·5 g/kg.

TABLE 6. ACUTE TOXICITY OF 5-IODODEOXYURIDINE IN MICE*

Amount of IUdR administered (g/kg)	Number of mice	Number of dead			
		24 hr	48 hr	72 hr	96 hr
None	10	None	None	None	None
2·5	13	8	None	None	None
2·00	11	1	1	None	None
1·25	10	None	None	1	None

*IUdR was suspended in 0·5 per cent carboxymethylcellulose in saline and each mouse received a single injection intraperitoneally (1·0 ml). The control mice were injected with the suspending media (1·0 ml).

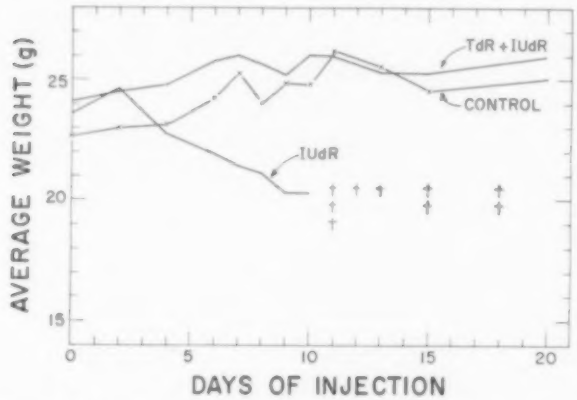

FIG. 5. Chronic toxicity of 5-iododeoxyuridine in mice and prevention by thymidine (see text for details).

In Fig. 5 is shown the results of a study of the chronic toxicity of IUdR in Swiss mice and the successful prevention of this toxicity by the prior administration of thymidine. Four groups of 10 mice were given daily intraperitoneal injections of 3, 6, 9 or 18 μmoles, respectively, of IUdR dissolved in water, while 4 additional groups were given 100 μmoles of thymidine prior to the administration of the stated amounts of IUdR; the control group was injected with saline. Only in the group which received the highest dose of IUdR, without thymidine, was loss of weight observed within a few days; on the 11th day of this dosage regimen 3 mice died. Half of the animals which received the high level of IUdR (18 μmoles per day) were dead by the 13th day and all had died by the 18th day. Thymidine prevented completely not only the lethal effects of IUdR, but also the loss of weight.

In Fig. 6 is shown a postulated scheme for the metabolism of IUdR. It is significant that no evidence for the formation of a primary dehalogenation product of IUdR was observed. If deoxyuridine were formed it must be very rapidly cleaved by nucleosidase to form free uracil. Because both iodouracil and uracil were observed the initial degradation step most probably was a direct cleavage of the iodinated deoxyribonucleoside with the formation of iodouracil.

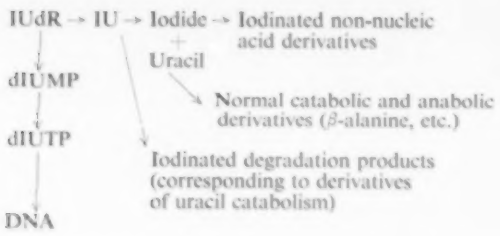
IUdR → IU → Iodide → Iodinated non-nucleic acid derivatives
Uracil
dIUMP
Normal catabolic and anabolic derivatives (β-alanine, etc.)
dIUTP
Iodinated degradation products (corresponding to derivatives of uracil catabolism)
DNA

FIG. 6. Proposed scheme for the anabolism and catabolism of 5-iododeoxyuridine.

Studies of the metabolism of a related compound, 5-bromouracil, in human as well as other mammalian species, have been made[20, 21] in which reduction was reported of the double bond in the 5,6-position of the pyrimidine ring, prior to dehalogenation and the formation of uracil. That a similar reduction of 5-iodouracil occurred in the mouse would appear probable, because of the similar characteristics of iodo- and bromo-uracil; however, definitive evidence for this has not as yet been obtained. Comparison of the metabolic derivatives of radioactive IUdR labeled with I^{131} and with tritium indicated a minimum of 4 products which contained both labels. Whether some of these correspond to acyclic iodinated degradation products of uracil remains to be determined.

Because of the many metabolic derivatives of IUdR formed in the mouse, it is difficult to relate with certainty the observed toxicity to either a single compound or the interference with a single metabolic reaction. Whether the toxic substances even contain iodine, cannot yet be stated positively. However, the prevention of the toxicity of IUdR by thymidine is strongly indicative of the presumptive mechanism of action. Normally, IUdR is rapidly metabolized and excreted in the urine, and other studies[6] have shown that cells utilize thymidine about 40-fold more effectively than IUdR, for the biosynthesis of DNA *in vivo*. Hence, one may visualize thymidine as saturating the sensitive enzyme surface for a sufficiently long period to permit the intracellular concentration of IUdR to be lowered, by catabolism and excretion, to a nontoxic level. The concentration of IUdR required to produce toxicity was very critical, since a decrease in the amount, administered chronically to the mouse, from 18 μmoles to 9 μmoles resulted in a striking inability of the analogue to produce any manifestations of toxicity.

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DISTURBANCE OF OXIDATIVE PHOSPHORYLATION BY AN ANTIBIOTICUM PRODUCED BY PSEUDOMONAS COCOVENENANS

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Abstract—The effect of a very toxic agent with antibiotic properties (bongkrekic acid), produced by *Pseudomonas cocovenenans*, on the oxidation of citric acid cycle intermediates catalyzed by heart muscle tissue has been investigated. It was found that the oxidation of pyruvate, α-ketoglutarate and malate was inhibited, whereas oxidation of succinate and β-hydroxybutyrate was stimulated. The phosphorylation, coupled with these oxidations proved to be even more sensitive to bongkrekic acid. The concentration required to produce these effects on isolated mitochondria is very low; in most cases 10^{-6} M is very effective.

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SEVERAL years ago Van Veen and Mertens[1, 2] discovered that large-scale food intoxications on Mid-Java (Indonesia) were caused by a hitherto unknown micro-organism, *Pseudomonas cocovenenans*, growing on "bongkrek", a product prepared by the natives from defatted coconut. The main toxic agent produced by this bacterium turned out to be an acid and was called bongkrekic acid. This substance could be extracted from cultures of *Pseudomonas cocovenenans* on defatted copra by the same investigators.[3]

The chemical structure of bongkrekic acid has been partially elucidated by Nugteren and Berends.[4] The bruto formula is $C_{29}H_{40}O_7$ and the substance was shown to be branched olefinic tricarboxylic acid with a tertiary methoxyl group in a δ-position with respect to one of the carboxylic acid groups. Seven double bonds are present forming at least two conjugated systems. Hydrogenated bongkrekic acid is no longer toxic. In acid solutions the unsaturated compound is very unstable and decomposes rapidly to non-toxic products.

The pharmacological properties of bongkrekic acid have been studied by van Veen and Mertens[5] and recently also by von Holt.[6] The toxicity was tested on several species (monkeys, rabbits, rats and pigeons) and the product found to be lethal in every instance. Administration of 2 mg/100 g body weight to rats caused death within 2–5 hr; 1 mg/100 g was tolerated, but the same dose when repeated after 48 hr proved lethal.

Oral administration of bongkrekic acid initially produces hyperglycaemia, soon followed by a severe hypoglycaemia. Intravenous injections of glucose give some relief but do not prevent death. It could be shown that just before death all glycogen had disappeared from liver and heart. The lactic acid content of the blood increases to two to three times the normal value.

Finally it was found[7] that bongkrekic acid also possesses antibiotic properties towards many micro-organisms (yeasts, bacteria and molds).

Based on these facts an investigation was set up to get some insight into the mode

of action of this remarkable substance. After the failure of some glycolytic enzymes (hexokinase, aldolase, isomerase, 3-phosphoglyceraldehyde dehydrogenase) to show any sensitivity to bongkrekic acid, attention was directed to the citric acid cycle. In preliminary experiments we used a homogenate of sheep heart muscle; later rat heart mitochondria were used as enzyme source. Finally the investigation was extended to include a soluble α-ketoglutaric acid oxidase preparation.

MATERIALS AND METHODS

Sheep heart was obtained at a local slaughterhouse; it was removed from the body about 15 min after death of the animal and chopped into pieces of a few g. It was transported to the laboratory cooled with solid carbon dioxide and stored at −25°C for some months without appreciable loss of activity. Homogenates were prepared with a Bühler high-speed blendor. One or two lumps of frozen tissue were thawed and blendored in the phosphate buffer described by Krebs and Eggleston[8] for 45 sec; the blendor vessel was cooled with melting ice.

Rat heart mitochondria were isolated as described by Cleland and Slater[9] in 0·25 M sucrose – 0·01 M versene, pH 7·4, and suspended in 0·25 M sucrose (neutralized to phenol red).

Oxygen uptake was followed in a Warburg apparatus (air as gas phase) at 37 or 30 °C. Inorganic ortho-phosphate was assayed by the FiskeSubbaRow method. Oxidative phosphorylation was measured by the disappearance of inorganic phosphate from the incubation medium with glucose plus hexokinase (prepared according to Berger *et al.*[10] and purified until step 4) as the trapping system for ATP.* One of two identically filled Warburg vessels was deproteinized by tipping in 0·5 ml 17·5% trichloroacetic acid from the side arm after an equilibration period of 10 min in the waterbath; this vessel was used to assay the inorganic phosphate concentration at zero time. With the other vessel oxygen uptake was measured until the reaction was stopped in the same manner; inorganic phosphate was measured in the deproteinized fluid.

Samples of bongkrekic acid were generously supplied by Dr. D. H. Nugteren (for preparation see [4]) as solutions in 2·5% bicarbonate. These preparations contained about 25 % saturated fatty acids with 6–10 carbon atoms, chiefly capronic acid. The concentration of the samples was polarimetrically or spectrophotometrically determined. For removal of bicarbonate the BA-solutions were acidified with 2 N sulphuric acid and, immediately after the formation of a white flocculent precipitate, extracted with peroxide-free ether. After a washing of the ethereal solution with water BA was extracted from it with small portions of a 2·5% solution of potassium phosphate. Residual ether was removed under vacuum until its smell no longer could be detected.

Cytochrome-*c* was prepared as described by Keilin and Hartree[11]; the last purification step consisted in column chromatography on Amberlite IRC-50 according to Margoliash.[12]

α-Ketoglutaric acid dehydrogenase and succinate activating enzyme (P-enzyme) were isolated, purified and tested for activity with the methods of Kaufman.[13] α-KG dehydrogenase contained 0·58 mg protein/ml and had a specific activity of 0·42 (specific

* The following abbreviations are used: ADP, ATP: adenosinedi- and tri-phosphate; GDP, GTP: guanosinedi- and tri-phosphate; IDP, ITP: inosinedi- and tri-phosphate; CoA: coenzyme A; DPN: diphosphopyridine dinucleotide; BOH: β-hydroxybutyric acid; DNP: 2:4-dinitrophenol; BA: bongkrekic acid; α-KG: α-ketoglutaric acid; P_i: inorganic ortho-phosphate.

activity is expressed as μmoles α-KG oxidized per min/mg protein). P-enzyme contained 2·9 mg protein/ml and had a specific activity of 0·54 (specific activity expressed as μmoles succinate activated per 30 min/mg protein at 37 °C).

The nitrogen content of the mitochondria suspensions was measured with a Kjeldahl method on semi-micro scale, using the apparatus described by Markham.[14]

RESULTS

1. *Preliminary experiments with sheep heart muscle homogenate*

The homogenates prepared from sheep heart muscle showed a considerable oxygen uptake in the absence of added substrate, most probably due to the oxidation of endogenous substrates such as glycogen.

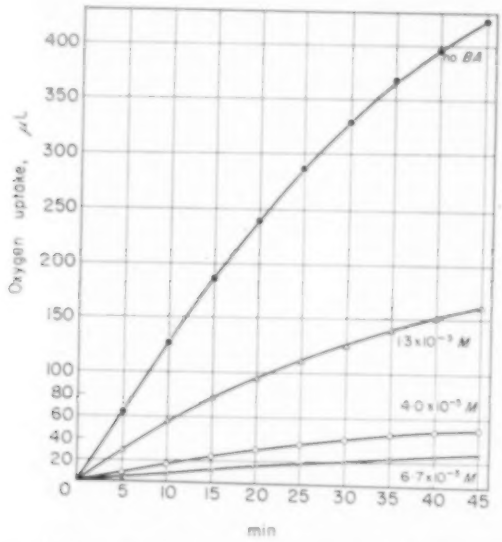

FIG. 1. Inhibition of oxidation of endogenous substrate by BA. A 12% heart muscle homogenate, reinforced with DPN^+ ($4·5 \times 10^{-4}$ M) and buffered at pH 7·4 was incubated at 37 °C with three concentrations of BA.

As may be seen from Fig. 1, this oxygen consumption is inhibited by BA and the inhibition depends on the concentration of the inhibitor.

When the oxygen uptake caused by the oxidation of endogenous substrate was inhibited by the addition of iodoacetic acid to about the same degree as with 0·003 M BA it appeared that the effects of these two inhibitors, when added together, were partially additive. This is shown in Table 1. From studies by Holzer *et al.*[15] it is known that the concentration of iodoacetic acid used will practically inhibit no enzyme of the glycolytic chain other than 3-phosphoglyceraldehyde dehydrogenase and as this enzyme is not sensitive to BA (unpublished experiments) it seemed profitable as a first approach to look for the point of attack of BA among those enzymes of carbohydrate metabolism operative beyond the triosephosphate stage.

Experiments designed to test the sensitivity of the citric acid cycle enzymes to BA were not very successful in consequence of the disturbing effect of the high endogenous oxygen consumption. Addition of citric or α-ketoglutaric acid did not affect the oxygen

TABLE 1. COMPARISON OF THE INHIBITORY EFFECT OF IODOACETIC ACID AND BONGKREKIC ACID ON THE OXYGEN CONSUMPTION OF HEART MUSCLE HOMOGENATE

Additions	Oxygen uptake (μl/45 min)	Inhibition (%)
Experiment 1		
none	198	0
iodoacetic acid $1{\cdot}6 \times 10^{-2}$ M	53	73
bongkrekic acid 3×10^{-3} M	53·5	73
iodoacetic acid + bongkrekic acid	24·5	87·5
Experiment 2		
none	165·5	0
iodoacetic acid $1{\cdot}4 \times 10^{-2}$ M	62·5	62
bongkrekic acid 3×10^{-3} M	55·5	66·5
iodoacetic acid + bongkrekic acid	27·5	83·5

Homogenate, 10% (exp. 1), 7% (exp. 2); DPN, $4{\cdot}5 \times 10^{-4}$ M; cytochrome *c*, 1 mg/ml.

uptake, whereas pyruvic acid reduced oxygen uptake considerably, this reduction being enhanced by BA. The addition of CoA however gave remarkable results. As is shown in Table 2 we found that in the presence of CoA BA had a less inhibitory effect on the O_2-uptake. CoA used in these experiments was a commercial preparation obtained from the Pabst-brewery (Milwaukee, Wisconsin, U.S.A.); it was about 75 % pure. The depression of the O_2-uptake, when large amounts of CoA (600 γ/ml) were added (experiment 3, Table 2) may presumably be ascribed to toxic impurities in this CoA sample.

TABLE 2. CoA INDUCED REVERSAL OF THE INHIBITORY EFFECT OF BONGKREKIC ACID ON THE OXYGEN CONSUMPTION OF HEART MUSCLE HOMOGENATE

Additions	O_2-uptake (μl/45 min)	Inhibition (%)
1. None	462·5	
BA: 3×10^{-3} M	85·5	81
CoA: 200 γ/ml	487·5	
BA + CoA	151	69
2. None	351	
BA: 3×10^{-3} M	63·5	82
CoA: 200 γ/ml	406	
BA + CoA	139·5	66
3. None	351	
BA: 3×10^{-3} M	63·5	82
CoA: 600 γ/ml	192	
BA + CoA (600 γ/ml)	103·5	46

Homogenate, 11% (exp. 1) and 8% (exp. 2 and 3); DPN, 6×10^{-4} M; cytochrome *c*, 1 mg/ml.

In the presence of CoA (200 γ/ml) the addition of citrate gave an extra O_2-uptake; this extra O_2-consumption was almost totally reduced by the addition of BA (Table 3). The oxidation of α-KG seemed not to be very sensitive to BA: the addition of α-KG even had some relieving effect, for the percentage inhibition dropped to about half that of the control at the highest concentration of α-KG used (Table 3). Further experiments however showed that this relieving effect of α-KG did not indicate an

insensitivity of the α-KG oxidase to BA, but that it was most probably caused by a small amount of succinate in the α-KG preparation, because in the presence of malonic acid the relieving effect of α-KG addition was completely absent (Table 3).

TABLE 3. THE EFFECT OF BONGKREKIC ACID ON THE OXIDATION OF CITRATE AND α-KG BY SHEEP HEART HOMOGENATE IN THE PRESENCE OF CoA

Additions	O_2-uptake (μl/hr)		Inhibition (%)
	No BA	BA present	
Exp. 1			
None	588	167	72
citrate	743	199	73
α-KG	653	305	53
Exp. 2			
None	473	146	69
α-KG	619	373	40
malonic acid	99	37	63
α-KG + malonic acid	323	108	66

All Warburg flasks contained $4{\cdot}5 \times 10^{-4}$ M DPN^+ and 200 γ/ml CoA. Further additions: citrate, $5{\cdot}6 \times 10^{-3}$ M; α-KG, $2{\cdot}4 \times 10^{-3}$ M (exp. 1) and $4{\cdot}4 \times 10^{-3}$ M (exp. 2); malonate, 5×10^{-3} M; BA, 3×10^{-3} M. In exp. 1 the concentration of the homogenate was 7·7% and in exp. 2, 8·4%; all measurements were made at 30 °C.

The oxidation of succinic acid however very clearly proved not to be impaired by BA (Fig. 2); oxygen uptake was considerably increased by the addition of succinate and an inhibition of only 10 % was found when BA was present.

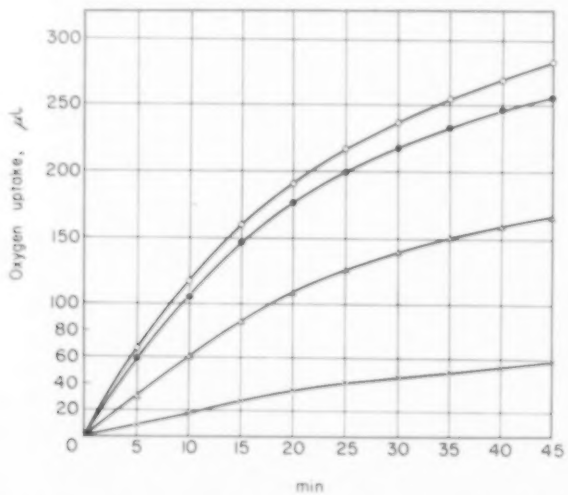

FIG. 2. Decrease of inhibition of oxygen uptake in the presence of succinate. To a heart muscle homogenate (7 per cent) reinforced with $4{\cdot}5 \times 10^{-4}$ M DPN^+ were added BA ($2{\cdot}5 \times 10^{-3}$ M) and succinate ($1{\cdot}2 \times 10^{-2}$ M). △-△-△-△, no addition; ○-○-○-○, succinate; ×-×-×-×, BA; ●-●-●-●, succinate + BA.

2. *Experiments with isolated rat heart muscle mitochondria*

The incubation medium for measuring O_2-uptake and oxidative phosphorylation

was based on that described by Slater and Cleland[16] and Slater and Holton.[17] Some minor modifications (halving of the fluoride and doubling of the Mg^{2+}-concentration) were found to be necessary to obtain optimum P/O-ratios in our hands. With α-KG P/O-ratios between 2·4 and 3·5 (mean 2·7 in 14 experiments) were obtained, and this value was practically independent of the incubation time between 10 and 40 min.

The effect of BA on the oxidative phosphorylation with α-KG as the substrate is shown in Table 4. In this table the results of 4 experiments covering an inhibitor range from 10^{-3} M to 10^{-6} M are summarized. The general conclusion from these results is that both oxygen uptake and phosphorylation are inhibited, but that phosphorylation is more sensitive to BA than oxidation. With increasing concentrations of the inhibitor rather curious results were obtained regarding its effect on the oxygen consumption:

(a) in contrast to what was to be expected the inhibition decreases rather than increases;
(b) the percentage of inhibition never reaches a value of 100 %
(c) the percentage of inhibition varies considerably from experiment to experiment and seems to be related to the amount of mitochondria (the higher the amount of mitochondria the smaller the inhibition).

TABLE 4. THE EFFECT OF BONGKREKIC ACID ON THE OXIDATIVE PHOSPHORYLATION IN RAT HEART MITOCHONDRIA WITH α-KETOGLUTARIC ACID AS SUBSTRATE

Conc. BA mmoles/ml	Decrease of inorganic P (μmole/mg N per hr)	O_2-uptake (μ atom/mg N per hr)	Inhibition of O_2-uptake (%)	P/O ratio
		Experiment 1		
—	147·7	60·5		2·44
1×10^{-3}	neg.	25·4	58	—
5×10^{-4}	neg.	19·2	68	—
1×10^{-4}	neg.	10·8	82	—
		Experiment 2		
—	152·6	54·6		2·79
1×10^{-4}	neg.	22·8	59	—
5×10^{-5}	neg.	19·7	64	—
1×10^{-5}	neg.	18·7	66	—
		Experiment 3		
—	137·6	38·8		3·55
1×10^{-5}	neg.	7·4	81	—
5×10^{-6}	neg.	6·7	83	—
1×10^{-6}	93·2	31·1	20	3·0
		Experiment 4		
—	190·7	59·4		3·21
$5{\cdot}3 \times 10^{-6}$	neg.	13·5	77	—
$3{\cdot}3 \times 10^{-6}$	neg.	19·5	67	—
$2{\cdot}0 \times 10^{-6}$	neg.	17·0	71	—
$6{\cdot}7 \times 10^{-7}$	173·7	62·3	−5	2·79

All vessels contained phosphate buffer, pH 7·4, 90 μmoles; malonate, 30 μmoles; NaF, 60 μmoles; $MgCl_2$, 30 μmoles; α-KG, 30 μmoles; glucose, 65 μmoles; ATP, 5 μmoles; cytochrome-*c*, 0·04 μmoles; versene, 6 μmoles; hexokinase, 0·05 ml; mitochondria, 1 ml; total volume, 3 ml. Concentration of mitochondria suspension in mg N/ml, exp. 1, 0·296; exp. 2, 0·395; exp. 3, 0·197; exp. 4, 0·258. All measurements were made at 30 °C.

An explanation of the first result (a) was sought in a contamination of the BA preparation with fatty acids. If these fatty acids should be oxidized by the mitochondria an increasing oxygen consumption could be expected with increasing concentrations of inhibitor, resulting in an apparent decrease of the percentage inhibition, the inhibition of the α-ketoglutaric acid dehydrogenase having reached its maximum value at a lower BA concentration. Incubation of BA in substrate amounts with mitochondria did not give oxygen uptake, but when oxaloacetic acid was added as a sparker for fatty acid oxidation an oxygen consumption proportional to the concentration of BA and consequently to the concentration of the contaminating fatty acids was observed (Table 5). The question, however, had then to be answered how sparking was

TABLE 5. OXIDATION OF FATTY ACIDS, PRESENT AS IMPURITIES IN BONGKREKIC ACID PREPARATIONS, SPARKED BY OXALOACETIC ACID

Added BA μmoles	Oxygen consumption (μl/mg N per hr)	
	5 μmole oxaloacetic acid	10 μmole oxaloacetic acid
0	44·4	82·1
1·7	74·2	199·8
3·4	108·2	240·0
5·1	178·1	—
6·8	169·6	236·5

The reaction medium contained phosphate buffer pH 7·4, 90 μmoles; NaF, 60 μmoles; $MgCl_2$, 30 μmoles; ATP, 5 μmoles; cytochrome-c, 0·04 μmoles; versene, 6 μmoles; mitochondria, 1 ml. Incubation temperature, 30 °C. The total volume of the reaction medium was 3·0 ml.

effected in the experiments with α-KG as a substrate, in which malonic acid was added to inhibit succinic acid dehydrogenase. If the concentration of malonic acid used was not large enough to give a complete inhibition of succinate oxidation, sparker formation might still occur and in that case an effect of the concentration of malonic acid on the inhibition-percentage should be demonstrable. This proved actually to be the case as is shown in Table 6; it also follows from this table that the percentage inhibition of the oxygen-uptake decreases with increasing incubation time (last column excepted). This is presumably the result of a steadily increasing concentration of sparker for fatty acid oxidation. When the concentration of malonic acid was raised to 40 μmole/ml a fairly constant inhibition-percentage was obtained between 10^{-3} M and 3×10^{-6} M BA (Table 7). The concentration range between zero and maximal effect of BA seems to be very narrow, as may be concluded from Table 4 (experiment 4) and Table 7 (experiment 1).

Experiments in which succinate was used as a substrate, showed that inhibition of oxygen uptake was not a general aspect of bongkrekic acid activity: the oxidation of succinate is considerably stimulated. In a typical experiment an increase of 65 % was found with a concentration of 3×10^{-6} M bongkrekic acid. Phosphorylation however is totally abolished even with the lowest concentration bongkrekic acid used (7×10^{-7} M). The stimulating effect on the succinate oxidation is in agreement with the results of the experiment with heart muscle homogenate, where the addition of succinate could repair the strongly inhibited oxidation of endogenous substrate (Fig. 2).

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TABLE 6. EFFECT OF MALONATE CONCENTRATION AND INCUBATION TIME ON THE PERCENTAGE INHIBITION OF α-KETOGLUTARIC ACID OXIDATION BY BONGKREKIC ACID

Incubation time in min	Inhibition of oxygen uptake (%)					
	Experiment 1			Experiment 2		
	No malonate	0·003 M malonate	0·02 M malonate	No malonate	0·01 M malonate	0·02 M malonate
10	54·4	59·4	72·7	61·6	69·7	72·5
20	53·2	56·1	68·5	59·7	66·9	76·8
30	53·1	53·6	67·2	57·6	64·4	76·9
40	50·8	52·0	65·8	54·6	61·1	76·9
50	45·3	49·3	63·4	47·6	49·0	71·9

The reaction medium consisted of phosphate buffer pH 7·4, 90 μmoles; NaF, 60 μmoles; versene, 6 μmoles; α-KG, 30 μmoles; $MgCl_2$, 30 μmoles; cytochrome-*c*, 0·04 μmoles; ATP, 5 μmoles; malonic acid as indicated; BA, 10^{-4} M; mitochondria, 1 ml. In experiment 1 the nitrogen content of the mitochondria suspension was 0·304 mg N/ml, in experiment 2, 0·465 mg N/ml. Incubation temperature, 30 °C.

TABLE 7. INHIBITION OF α-KETOGLUTARIC ACID OXIDATION BY VARIOUS CONCENTRATIONS OF BONGKREKIC ACID IN THE PRESENCE OF A HIGH CONCENTRATION OF MALONIC ACID

Conc. BA (mmoles/ml)	Experiment 1		Experiment 2	
	Oxygen uptake (μl/mg N per hr)	Inhibition (%)	Oxygen uptake (μl/mg N per hr)	Inhibition (%)
0	470	0	471	0
6×10^{-7}	477	−2	—	—
1×10^{-6}	463	2	—	—
$1{\cdot}5 \times 10^{-6}$	436	7	—	—
3×10^{-6}	227	52	—	—
6×10^{-6}	208	56	—	—
1×10^{-5}	196	59	224	52
3×10^{-5}	208	56	—	—
5×10^{-5}	—	—	220	53
1×10^{-4}	204	57	215	54
5×10^{-4}	—	—	216	54
1×10^{-3}	215	54	206	56

Composition of incubation medium see Table 6. Mitochondria concentration, exp. 1, 0·487 mg N/ml; exp. 2, 0·480 mg N/ml. Incubation temperature, 30 °C.

Pyruvic acid was rapidly oxidized by the heart muscle mitochondria in the presence of a small amount of succinate; this oxidation however was impaired when BA was added to the reaction medium. This is shown in Fig. 3.

The oxidation of α-ketoglutaric acid and pyruvic acid shows a great analogy: both substrates are oxidized via an acylcoenzyme A intermediate, and the hydrogen is transferred via lipoic acid to DPN. In the oxidation of succinate DPN is not involved. Therefore it seemed of interest to investigate the effect of BA on the oxidation of those intermediates of the tricarboxylic acid cycle where the hydrogen atoms are transferred via DPN, but where no acylcoenzyme A intermediate is involved. Malic acid fulfils this condition and it was found that BA inhibits the oxidation of this tricarboxylic acid cycle intermediate by heart muscle mitochondria.

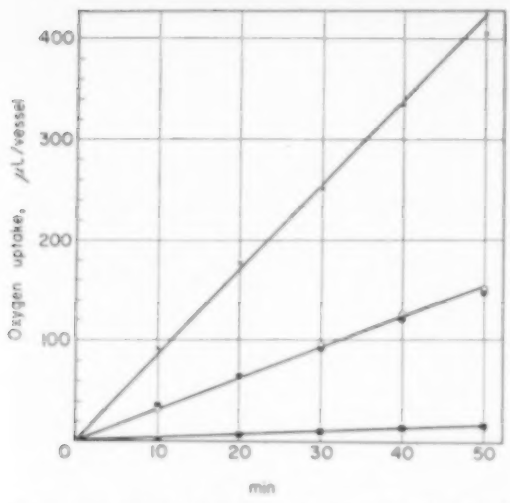

FIG. 3. Inhibition of pyruvate oxidation by BA. All Warburg vessels contained phosphate buffer pH 7·4, 90 µmoles; NaF, 60 µmoles; $MgCl_2$, 30 µmoles; cytochrome-*c*, 0·04 µmoles; versene, 6 µmoles; mitochondria 1·0 ml (0·598 mg N/ml); ATP, 5 µmoles. ■–■–■–■, succinate, 2 µmoles; ×–×–×, succinate + pyruvate (20 µmoles); ●–●–●–●, succinate + pyruvate (20 µmoles) + BA (5×10^{-6} M); ○–○–○–○, succinate + pyruvate (20 µmoles) + BA (1×10^{-5} M).

Further experiments were performed with the immediate precursor of malic acid in the cycle fumaric acid. The oxidation of fumaric acid and also the phosphorylation coupled with this oxidation proved to be very sensitive to BA. Concentrations of inhibitor as low as $6{\cdot}7 \times 10^{-6}$ M already give an inhibition of the oxygen uptake of about 40 % but there is a considerable variation in the amount of inhibition between single experiments, probably related to the mitochondria concentration.

TABLE 8. INHIBITION OF FUMARATE OXIDATION BY BONGKREKIC ACID

Conc. BA mmoles/ml	Inhibition (%)	
	Experiment 1	Experiment 2
$1{\cdot}3 \times 10^{-6}$	2	—
4×10^{-6}	39	—
$6{\cdot}7 \times 10^{-6}$	38	42
$1{\cdot}1 \times 10^{-5}$	42	—
$1{\cdot}3 \times 10^{-5}$	42	48·5
2×10^{-5}	48	51
$2{\cdot}7 \times 10^{-5}$	71	57
4×10^{-5}	—	66
$5{\cdot}4 \times 10^{-5}$	—	68
$6{\cdot}7 \times 10^{-5}$	—	72

Composition of incubation medium phosphate buffer pH 7·4, 90 µmoles; NaF, 60 µmoles; versene, 6 µmoles; $MgCl_2$, 30 µmoles; fumarate, 30 µmoles; ATP, 5 µmoles; cytochrome-*c*, 0·04 µmoles; mitochondria, 1 ml; total volume, 3 ml. Incubation temperature, 30 °C. Mitochondrial concentration, 0·463 mg N/ml (exp. 1) and 0·512 mg N/ml (exp. 2).

In Table 8 the results of two experiments are summarized, covering a BA-concentration range of from $1{\cdot}3 \times 10^{-6}$ to $6{\cdot}7 \times 10^{-5}$ M. Phosphorylation is totally abolished at

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BA-concentrations higher than $6{\cdot}7 \times 10^{-6}$ M. At lower concentrations there is no clear relation between inhibition of O_2-uptake and phosphorylation, as may be seen from Table 9. At the lowest concentration a small rise in the P/O-ratio can be observed, but when the BA-concentration is doubled a further increase in P/O-ratio is found in one experiment and a decrease in the other.

TABLE 9. INHIBITION BY BONGKREKIC ACID OF OXIDATIVE PHOSPHORYLATION WITH FUMARATE AS SUBSTRATE

Conc. BA mmoles/ml	Experiment 1			Experiment 2		
	Percentage inhibition of		P/O ratio	Percentage inhibition of		P/O ratio
	Oxygen uptake	Phosphorylation		Oxygen uptake	Phosphorylation	
$6{\cdot}7 \times 10^{-7}$	31	22	1·96	12	6	1·92
$1{\cdot}3 \times 10^{-6}$	33	8	2·24	17	24	1·66
$6{\cdot}7 \times 10^{-6}$	60	70	1·22	52	100	—

Composition of incubation medium, see Table 8; in these experiments were also added hexokinase, 0·05 ml and glucose, 65 μmoles to each vessel. Mitochondrial concentration, 0·593 mg N/ml (exp. 1) and 0·426 mg N/ml (exp. 2). P/O ratios without BA, 1·65 (exp. 1) and 1·81 (exp. 2).

Finally some experiments were done to investigate the effect of BA on the oxidation of β-hydroxybutyrate by heart muscle mitochondria. No concomitant phosphorylation was measured and glucose plus hexokinase were therefore left out of the medium. The oxidation of this substrate was found to be very slow and it could not be enhanced by adding small amounts of succinate. This is shown in Fig. 4: the oxygen uptake of BOH + succinate (curve D) is equal to the sum of oxygen uptakes when BOH or succinate are added separately (curve A and B). BA has only a very small inhibitory effect on the oxidation of BOH alone (curve C), but a very pronounced stimulation of oxygen consumption is obtained in the presence of succinate (curve E and F).

3 *Experiments with isolated α-KG oxidase*

The enzymic oxidation of α-KG to succinic acid in mitochondria is a rather complex one. Through the work of Kaufman *et al.*[18, 19] and Sanadi *et al.*[20–24] it is known that the reaction sequence can be written as follows:

(1) α-KG + CoA-SH + DPN^+ ⟶ succinyl-S-CoA + CO_2 + DPNH + H^+
(2a) Succinyl-S-CoA + P_i + GDP ⟶ succinate + CoA-SH + GTP
(2b) GTP + ADP ⟶ GDP + ATP
(3) Succinyl-S-CoA + H_2O ⟶ succinate + CoA-SH.

All these reactions are catalyzed by separate enzymes; α-KG dehydrogenase for reaction (1), GDP phosphorylation enzyme for reaction (2a). Reaction (2b) is catalyzed by a diphosphonucleosidekinase (NDP-kinase) and reaction (3) by succinyl-CoA deacylase. GDP phosphorylation enzyme and NDP-kinase together have been called P-enzyme by Kaufman.[18] Isolated α-KG dehydrogenase contains bound thiamine-pyrophosphate and α-lipoic acid, the latter is most probably bound on the enzyme as the acid amide[25] and plays a role not only in electron transport between substrate and DPN^+ but also in acylgroup transfer to CoA.[26] Reaction (2a) seems to be the sum of

at least two reactions, since Smith *et al.*[27] could show that in *E. coli* succinate activation (reverse of reaction) proceeds via phosphoryl-S-CoA; it remains to be demonstrated that this intermediate is also involved in animal α-KG oxidase. IDP and ITP can replace GDP and GTP in reactions (2a) and (2b), as was shown by Sanadi *et al.*[24]

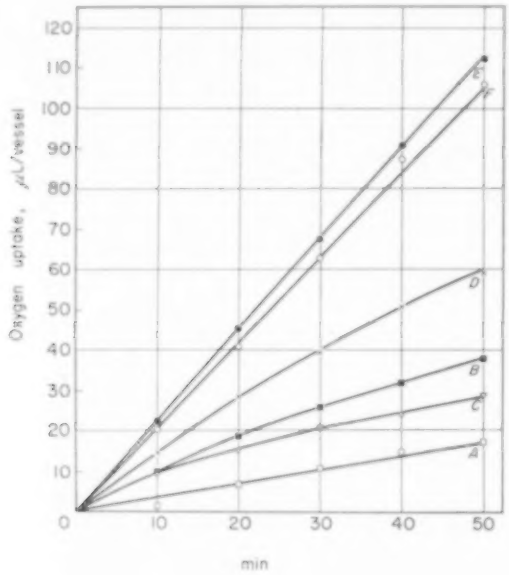

FIG. 4. Stimulation of the oxidation of β-hydroxybutyric acid by bongkrekic acid in the presence of small amounts of succinate. The vessels contained: phosphate buffer, pH 7·4, 90 μmoles; NaF, 60 μmoles; $MgCl_2$, 30 μmoles; cytochrome-*c*, 0·04 μmoles; versene, 6 μmoles; ATP, 5 μmoles; mitochondria, 1 ml and when present succinate, 2 μmoles; BOH, 40 μmoles.
Curve A: succinate, curve B: BOH. curve C: BOH + BA (10^{-5} M), curve D: BOH + succinate, curve E: BOH + succinate + BA (5×10^{-6} M), curve F: BOH + succinate + BA (10^{-5} M). Mitochondrial suspension contained 0·598 mg N/ml. Temperature, 30 °C.

When α-KG dehydrogenase was tested on its sensitivity to BA no inhibition was found, but on coupling α-KG dehydrogenase with P-enzyme an inhibition of enzymic activity was clearly demonstrated; the concentration of BA however necessary to demonstrate inhibition was very high: $1{\cdot}2 \times 10^{-3}$ M gave a decrease of 58 % of the control value (Fig. 5, curve A). When ADP was left out of the incubation medium, the oxidation velocity of α-KG was lower, but still sensitive to BA (curve B); addition of BA at the beginning of the experiment gave an initial reaction velocity, that was equal to that after BA addition at 22 min, but levelled off to zero after about 20 min (curve C). When no ADP is added reaction (2b) does not progress; as inhibition was found in this case too, it seems probable that BA attacks on the GDP (IDP) phosphorylation enzyme rather than on the NDP kinase. This can also explain the fact that with mitochondria a total inhibition of α-KG oxidation was never found: if it may be assumed that the breakdown of succinyl-CoA via reaction (3) is not impaired, oxidation of α-KG to succinate remains possible.

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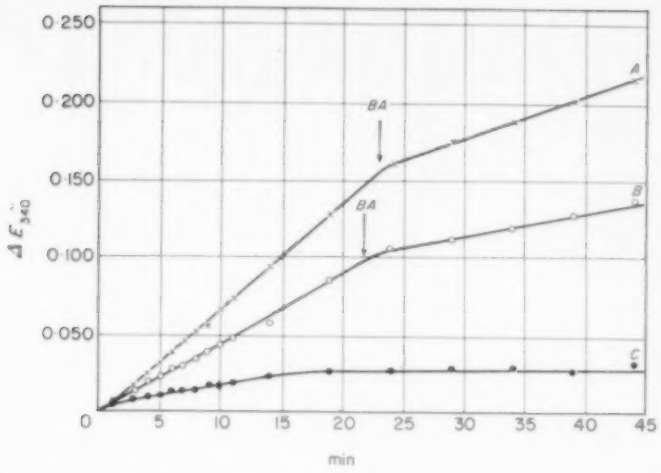

FIG. 5. Inhibitory effect of bongkrekic acid on purified P-enzyme. Beckman spectrophotometer culvettes contained 100 μmole Trisbuffer pH 7·4; 30 μmoles potassium phosphate pH 7·4; 7·5 μmoles $MgCl_2$; 0·22 μmoles DPN; 40 γ CoA; 8·3 μmoles cysteine; 25 μmoles α-KG; 1·8 μmoles IDP; 1·6 μmoles ADP; 0·2 ml α-ketoglutaric dehydrogenase; 1·0 ml P-enzyme; water to 3 ml. The cysteine was neutralized just before starting the assay. Curve A: complete reaction mixture, BA added after 23 min; curve B and C: ADP omitted from the reaction mixture, BA added after 22 min (B) or before addition of enzyme (C).

DISCUSSION

This investigation reveals that BA, must be considered as a potent inhibitor of mitochondrial enzymes. The process of oxidative phosphorylation—the energy generating mechanism in the oxidative breakdown of the carbohydrates and fats—is strongly impaired by BA, and this must lead to a lack of "energy rich" triphosphates in the BA sensitive organs.

Several phenomena that are found on BA intoxication can be explained by this general picture of the action of BA. As the phosphorylation of ADP coupled with the oxidation of 3-phosphoglyceraldehyde is the only way open to form ATP when the tricarboxylic acid cycle is blocked, it is understandable that stocks are mobilised, i.e. that glycogen disappears from liver, heart and muscle; that the glucose content of the blood is initially increased, but decreases as soon as the glycogen is exhausted; and that lactic acid will accumulate. Von Holt[6] found a very sudden appearance of shock after administration of BA to rats, which might perhaps be ascribed to heart insufficiency caused by a shortage of ATP required for performing work. In advanced cases of BA intoxication patients get in coma, which may be result of disturbance of oxidative phosphorylation in the brain.

This study sheds no light on the precise mode of action of this toxic agent; only points of attack are located. We do not know for example whether BA affects the structural integrity of the mitochondria or whether its activity may be due to inhibition of "non-structure-related" mitochondrial reactions. The first possibility seems a more plausible one, because the concentration which is effective on soluble α-KG oxidase is many times greater than with mitochondria.

The effect of BA on the oxidative capacity of mitochondria is dimorphic: the oxidation of pyruvate, α-KG and malate is inhibited, that of succinate and BOH is stimulated. In the experiments with BOH a racemic mixture of the D(−) and L(+)-form was used. As is shown by Beechey[28] both isomers are not oxidized in the same manner by rat heart mitochondria. For the oxidation of D(−)-BOH to water and carbon dioxide no catalytic amount of citric acid cycle intermediates is required as sparker, in contrast to that of L(+)-BOH, which showed a need for small quantities of fumarate, and proved to be sensitive to fluoride. Uncoupling agents like DNP and dicoumarol inhibited the oxidation of both isomers. This situation is in a sharp contrast to what is known of the oxidation of BOH by liver mitochondria. Lehninger and Greville[29] found that the sole oxidation product of D(−)-BOH was acetoacetic acid, whereas L(+)-BOH gave chiefly citric acid and only small amounts of acetoacetic acid in the presence of sparker. DNP stimulates the oxidation of BOH by liver mitochondria, as was shown by Judah.[30] In our case no promotion of oxygen uptake was found on addition of succinate; a possible explanation may be that the fluoride concentration we used was so high that sparking of L(+)-BOH oxidation was no longer possible, but then it is not understandable why the stimulation of oxygen uptake was found in the presence of succinate only. Appropriate controls ruled out the possibility that the extra oxygen consumption was due to an effect of BA on succinate and not on BOH; moreover, stimulation was also found when not succinate but oxaloacetate was added in sparking amounts. This question cannot be solved before more experimental evidence has been obtained.

The fact that BA only stimulates the oxidation of succinate and BOH may be connected with the findings of Lehninger and Cooper[31] and of Kielley and Bronk[32] who prepared fragments of mitochondria, carrying part of the oxidative and phosphorylative capacities of whole mitochondria. The only dehydrogenases still present in these particles are those for BOH and succinate and this points to a close spatial arrangement of these dehydrogenases in the intact mitochondria. This may be used as another argument for the hypothesis that BA acts primarily on the mitochondrial structure.

BA can be considered as a new type of uncoupler of oxidative phosphorylation; its structure (as far as it is known at this moment) is very uncommon and it differs principally from all other known uncouplers in that its unihibitory power includes the substrate-linked phosphorylation of α-KG.

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TOXIC ACTION OF ORGANOPHOSPHORUS COMPOUNDS AND ESTERASE INHIBITION IN HOUSEFLIES

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Abstract—The paper deals with investigations on the inhibition *in vivo* of the cholinesterase and the aliesterase in houseflies poisoned by treatment with organophosphorus insecticides. The kinetics of the inhibition of esterases by DDVP, paraoxon and diazoxon in the presence and in the absence of substrate were closely studied. It was found that the inhibition is strongly retarded by the presence of acetylcholine in the case of the cholinesterase, and of phenylbutyrate in the case of the aliesterase. It could thus be shown that the addition of these substrates at the time of homogenization lends appropriate protection to the enzymes against free inhibitor present in the body of the insect. The results obtained with this "protective method" show that cholinesterase inhibition at the time of knock-down was generally less than 50 per cent, whereas the aliesterase inhibition at this time proved to be about 90 per cent. It could further be shown that the *in vivo* aliesterase inhibition increased rapidly from the beginning of exposure of the flies to the insecticide, and reached values of more than 50 per cent even long before knock-down occurred. This inhibition was found to be not readily reversible *in vivo*.

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ORGANOPHOSPHORUS compounds have been shown to be potent inhibitors of cholinesterases,[1, 2, 3, 4] a number of other esterases and lipases[5] and also of some proteolytic enzymes.[6, 7] Further investigations on the action of these substances *in vitro* and *in vivo* left little doubt that their toxicity in mammals is due to the inhibition of the cholinesterase in the nervous system. Strong arguments have been put forward that the toxic action of the insecticidal organophosphates is also due to the inhibition of the cholinesterase in the insect,[8] although the correctness of this view has also been doubted by a number of authors (see Spencer and O'Brien[9]).

Houseflies contain two enzymes that are highly sensitive to organophosphates: a cholinesterase and an enzyme indicated as aliesterase.[10] The latter exerts strong hydrolytic activity on methyl- and ethylbutyrate and some other aliphatic esters, and also on phenylbutyrate and other aromatic esters. The present paper deals with investigations on the reaction of organophosphates with these fly-esterases *in vitro* and *in vivo*, in which special attention is given to the difficulties encountered in obtaining reliable *in vivo* inhibition data. The purpose of this study is mainly to establish a relation between the biochemical symptoms, i.e. the esterase inhibition, and the externally visible symptoms of intoxication.

METHODS AND MATERIAL

Measurement of esterase-activity was performed by the Warburg manometric method. The reaction medium contained 0·025 M $NaHCO_3$, the gas phase consisted

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of 95% N_2 and 5% CO_2, pH was about 7·5. Cholinesterase activity was measured at 37 °C and aliesterase activity at 27 °C. In view of the lability of the aliesterase, especially in alkaline media, homogenates for the estimation of this enzyme were prepared in ice-cold saline, and bicarbonate was added at the very last moment before measurement started. The substrates used were acetylcholine (0·015 M) for cholinesterase and methylbutyrate and phenylbutyrate for aliesterase determinations. Because of its low water-solubility, phenylbutyrate was always used in the form of an emulsion (0·025 ml phenylbutyrate per Warburg flask in most cases; a small amount of emulsifier was added).

The organophosphorus compounds used were DDVP = *O* : *O*-dimethyl *O*-2 : 2 dichlorovinyl phosphate; Parathion = *O* : *O*-diethyl-*O*-*p*-nitrophenyl phosphorothionate; Paraoxon = *O* : *O*-diethyl-*O*-*p*-nitrophenyl phosphate; Diazinon = *O* : *O*-diethyl-*O*-(2-*iso*propyl-4-methyl-6-pyrimidinyl) phosphorothionate; Diazoxon = *O*-*O*-diethyl-*O*-(2-*iso*propyl-4-methyl-6-pyrimidinyl) phosphate; Co-ral ≡ Bayer 21/199 = *O* : *O*-diethyl-*O*-(3-chloro-4-methyl-umbelliferone) phosphorothionate.

Insect material. The experiments were done with a normal insecticide susceptible strain of the housefly, *Musca domestica* L. The flies were reared on a milk powder–yeast diet at 25 °C and *c.* 70 per cent relative humidity. The flies were poisoned by exposing them to a residue of the toxicant on glass, or by exposing them to the vapour of the poison (in the case of DDVP and diazinon). The doses used varied a great deal and were chosen in such a way that knock-down (paralysis) of the flies occurred after 0·5–2 hr in most cases. Further particulars about exposure and the time course of the intoxication will be given where they are relevant.

RESULTS

1. *The inhibition of esterases by organophosphates* in vitro

The organophosphates studied were found to be potent inhibitors of both the cholinesterase and the aliesterase present in housefly homogenates. Table 1 shows some approximate figures with respect to the rate constants observed. An important

TABLE 1. BIMOLECULAR RATE CONSTANTS OF INHIBITION*
(l. moles^{-1} min^{-1})

	Cholinesterase† 37 °C	Aliesterase‡ 27 °C
DDVP	3×10^7	1×10^7
Paraoxon	6×10^6	8×10^7
Diazoxon	2×10^8	4×10^7

* The bimolecular rate constants have been calculated from the equation $v = k'.[E].[I]$, assuming that $Ki \gg [I]$, since then $[E] = [E_{tot}]$.

† Data obtained with fly head homogenates, made up in bicarbonate–Ringer.

‡ Data obtained with whole fly homogenates, made up in 1% NaCl.

point which deserves consideration is the specificity of the inhibitors for the esterases studied. This is reflected in the I_{50}-values (concentrations of the inhibitor giving 50 per cent inhibition after an "infinite" time of incubation), shown in Table 2. The

TABLE 2. CONCENTRATIONS AND AMOUNTS OF ORGANOPHOSPHATE GIVING 50 PER CENT INHIBITION OF ESTERASE ACTIVITY IN WHOLE FLY HOMOGENATES (ONE FLY PER FLASK)

	Cholinesterase		Aliesterase	
	(moles/l.)	(ng/fly)*	(moles/l.)	(ng/fly)*
DDVP	$4{\cdot}5 \times 10^{-9}$	2·0	3×10^{-9}	1·3
Paraoxon	6×10^{-9}	3·3	3×10^{-9}	1·7
Diazoxon	2×10^{-9}	1·2	$3{\cdot}5 \times 10^{-9}$	2·0

* 1 ng = 1×10^{-9} g.

data in the Tables 1 and 2 indicate that the three organophosphates studied react rapidly and rather specifically with the esterases under investigation.

The phosphorothionates do not inhibit esterase-activity *in vitro*, but according to the generally accepted opinion they are converted to their oxygen analogues in the animal and insect body and will thus cause inhibition *in vivo*.

2. *The measurement of* in vivo *inhibition*

If houseflies are exposed to an organophosphate the toxicant will penetrate into the body. Phosphorothionates will, after penetration, be oxidized to the corresponding phosphates. A part of the inhibitor thus absorbed or formed will react with and thus inactivate the esterases, another part will possibly react with other compounds in the body or be enzymically broken down, but in most cases free inhibitor will also be present. In our experiments the amount of free inhibitor generally does not exceed 10 ng per fly, and in most cases is considerably less. However, an amount of 5 ng per fly gives a concentration of about 10^{-6} M in the insect body and of approximately 10^{-8} M in the homogenates prepared for the manometric determination of the esterase activity. Such concentrations are likely to cause quick and strong inhibition. It must be assumed that structural barriers in the insect prevent or at least slow down the reaction between esterases and organophosphates. No such barriers, however, exist after homogenization and inhibition will thus rapidly proceed under these conditions. The presence of free inhibitor thus constitutes a serious difficulty in these measurements.

Kewitz,[11, 12] investigating the *in vivo* cholinesterase inhibition in mouse brain, extracted the tissue with choloroform in order to remove any free organophosphate present. The inhibition values so obtained were indeed considerably lower than those obtained without chloroform-extraction. Another method of preventing the reaction between free enzyme and free inhibitor during and after homogenization, consisting of the addition of substrate to the homogenizing medium,[13, 14, 15] will be discussed below.

The inhibitory reaction may be depicted as occurring in two steps:
a reversible one

$$E + I \rightleftharpoons EI, \quad K_i = \frac{[E] \times [I]}{[EI]} \tag{1}$$

and an irreversible one

$$EI \longrightarrow \text{phosphorylated enzyme} \quad v = k.\,[EI] \tag{2}$$

The rate at which irreversible inactivation of the enzyme occurs (v) will be proportional to the concentration of *EI*. Added substrate will compete with the inhibitor for the enzyme by the reaction

$$E + S \rightleftharpoons ES, \quad K_m = \frac{[E] \times [S]}{[ES]} \tag{3}$$

and thus lower the concentration of *EI*. From Lineweaver and Burk's equations[16] for the case of competitive inhibition it can be derived that

$$\frac{1}{v_{is}} = \frac{K_i}{V_{im}} \cdot \left(1 + \frac{[S]}{K_m}\right) \cdot \frac{1}{[I]} + \frac{1}{V_{im}} \tag{4}$$

and

$$\frac{1}{v_i} = \frac{K_i}{V_{im}} \cdot \frac{1}{[I]} + \frac{1}{V_{im}} \tag{5}$$

where v_{is} = velocity of inhibitory reaction in the presence of substrate;
v_i = velocity of inhibitory reaction in the absence of substrate;
K_i = dissociation constant of enzyme–inhibitor complex;
K_m = dissociation constant of enzyme–substrate complex;
V_{im} = maximal rate of inhibitory reaction, i.e. if all the non-phosphorylated enzyme is present as *EI*;
$[S]$ = substrate concentration;
$[I]$ = inhibitor concentration.

From equations (4) and (5) it follows that

$$\frac{v_{is}}{v_i} = \frac{K_i + [I]}{K_i([S] + K_m)/K_m + [I]} \tag{6}$$

No data are available on the value of K_i. Two borderline cases may be considered: the first, where

$$K_i \gg [I], \quad \text{then} \quad \frac{v_{is}}{v_i} \simeq \frac{K_m}{[S] + K_m} \tag{7}$$

$$\text{or} \quad \frac{v_{is}}{v_i} \simeq 1 - \frac{v}{V_{\max}} \tag{8}$$

where v = velocity of enzymic reaction in the absence of inhibitor, and $V_{\max}$ = maximal rate of enzymic reaction (at infinite substrate concentration); and the second, where

$$K_i \ll [I], \quad \text{then, unless } [S]/K_m \text{ is very high,}$$

$$\frac{v_{is}}{v_i} \simeq 1$$

Three enzyme–substrate systems, used in our studies on inhibition *in vivo*, will be briefly considered below. In the calculations it will be assumed that $K_i \gg [I]$, so that the equations (7) or (8) can be used. It is, however, not at all certain that this assumption is right. The values for v_{is}/v_i should therefore be taken as minimum values.

Housefly cholinesterase with acetylcholine as a substrate. The value of K_m for this system is not exactly known. According to some data on enzyme activity obtained with low acetylcholine concentrations and using the colorimetric method described by Hestrin[17], $K_m \leqq 10^{-4}$. This means that the presence of 10^{-2}M acetylcholine will strongly decrease the rate of the inhibitory reaction with organophosphates. If $K_m = 10^{-4}$ we obtain:

$$\frac{v_{is}}{v_i} = \frac{K_m}{[S] + K_m} = \frac{10^{-4}}{10^{-2} + 10^{-4}} = \frac{1}{101}$$

Housefly aliesterase with methylbutyrate as a substrate. Fig. 1 shows the *pS*-activity curve obtained for this system. According to this v/V_{max} at a methylbutyrate concentration of 0·12 M (vertical dotted line *P*) is approximately 0·85 and $v_{is}/v_i = 1 - (v/V_{max}) = 0{\cdot}15$. The rate of the inhibitory reaction will thus be only moderately reduced by the addition of substrate. It is impossible to improve the protection of the enzyme by increasing the methylbutyrate concentration because of the limited solubility of this compound in water (vertical dotted line *Q*).

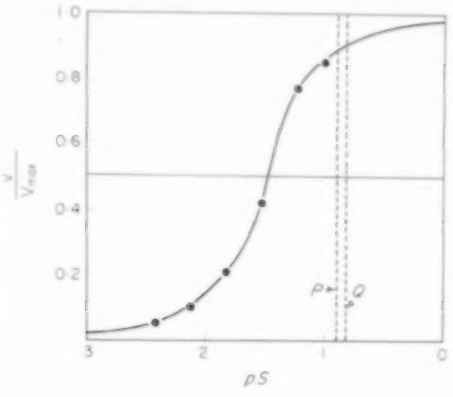

FIG. 1. Activity-*pS* curve for the hydrolysis of methylbutyrate by housefly-aliesterase. *P* indicates the *pS* at which *in vivo* measurements were performed. *Q* indicates the limit of solubility of methylbutyrate in water.

Housefly aliesterase with phenylbutyrate as a substrate. It has been shown[18] by substrate summation experiments that phenylbutyrate is hydrolysed by the same enzyme as methylbutyrate. Although this enzyme proved to be highly active against phenylbutyrate and other aromatic esters, the name aliesterase, chosen on account of its hydrolytic activity to methylbutyrate and other aliphatic esters, was maintained.

As it was found that the addition of methylbutyrate did not appropriately protect the aliesterase from inhibition by paraoxon and diazoxon (see below), some experiments were performed in which phenylbutyrate was used as the protecting substrate. The prospects of getting convenient results with this substrate were rather bad. As a consequence of its low solubility in water, $[S]$ was necessarily low. Only if the value of K_m would be extremely low, so that $[S]/K_m$ would still be high, good protection could be expected. No data on K_m were, however, available.

Whether sufficient protection of the enzyme against free inhibitor will be obtained by the addition of a substrate is dependent on many factors, viz. the $[S]/K_m$ value of the enzyme–substrate system, the $[I]/K_i$ value of the enzyme–inhibitor system, and the value of the reaction constant k of equation (2) (it should be emphasized that this k-value is not the bimolecular rate constant k' given in Table 1; actually $k = k' \cdot K_i$). Many of these factors are not or inexactly known. Therefore, experiments were performed to determine the extent to which protection occurs.

The esterases under investigation were mixed with their substrates and their activity measured by the manometric method. After about 20 min the inhibitor was added from a side arm of the Warburg flask, and measurement of enzyme activity continued for about 2 hr. The results are shown in the Figs. 2, 3 and 4.

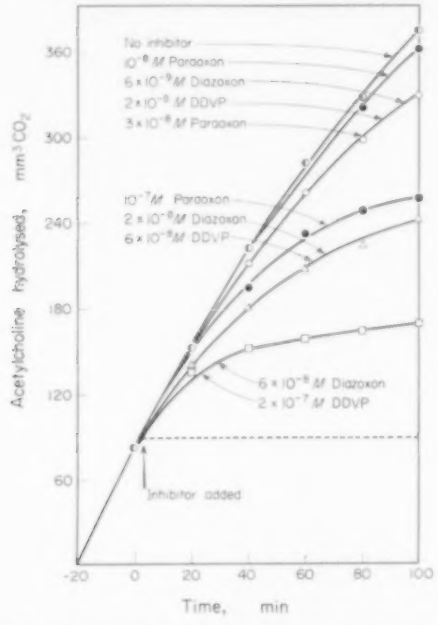

FIG. 2. Inhibition of housefly cholinesterase in the presence of the substrate acetylcholine (ACh). Initial ACh-concentration: 0·015 M; homogenate: two fly heads/flask; temp. 37 °C.

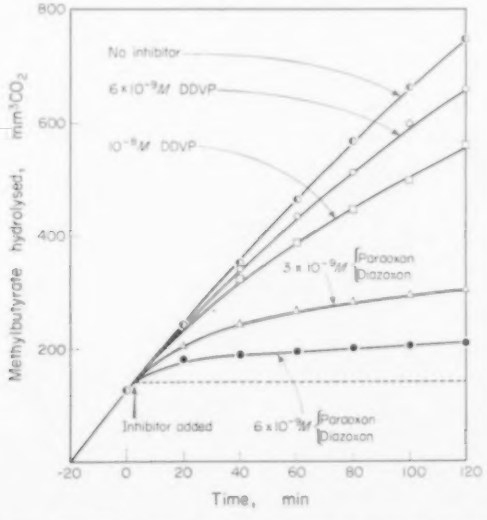

FIG. 3. Inhibition of housefly aliesterase in the presence of the substrate methylbutyrate (MB). Initial MB-concentration: 0·12 M; homogenate: 0·5 flies/flask; temp. 27 °C.

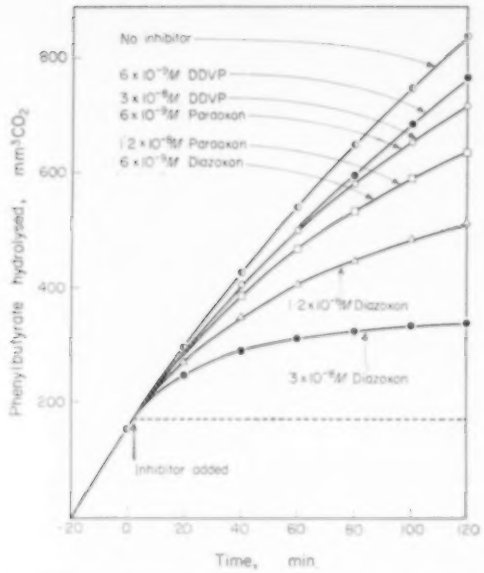

FIG. 4. Inhibition of housefly aliesterase in the presence of the substrate phenylbutyrate (PB). PB-emulsion (1 ml/flask): 1 ml PB + 0·2 ml emulsifier + 50 ml NaCl 1 per cent. Homogenate: 0·5 flies/flask; temp. 27 °C.

The following conclusions can be drawn:

(*a*) According to the data given in the Tables 1 and 2, DDVP, paraoxon and diazoxon in all concentrations used in these experiments would rapidly cause nearly complete inhibition of esterase activity in the absence of substrate.

(b) The addition of acetylcholine yielded very good protection of the cholinesterase against DDVP and paraoxon; much poorer protection was obtained against diazoxon (Fig. 2). This is in agreement with the very high rate constant for the reaction between cholinesterase and diazoxon. In the experiments on *in vivo* inhibition of the cholinesterase the conditions were such that the presence of 5 ng of organophosphate per fly would cause a concentration of about $1{\cdot}5 \times 10^{-8}$ M (1·5 flies per Warburg flask).

(c) Methylbutyrate did not appropriately protect the aliesterase from inhibition by paraoxon and diazoxon, but did so quite well in the case of DDVP (Fig. 3). Although some difference was anticipated from the difference in the bimolecular rate constants (Table 1), it was so striking that this experiment was repeated several times. Similar results were obtained throughout. *In vivo* aliesterase inhibition was measured under such conditions that 5 ng per fly would give rise to a concentration of about 5×10^{-9} M (0·5 flies per Warburg flask). In the case of DDVP the protection by methylbutyrate is probably sufficient.

(d) Very satisfactory results were obtained with phenylbutyrate (Fig. 4). The protection obtained decreased in the order DDVP > paraoxon > diazoxon. The interpretation of these experiments is complicated by the fact that approximately 10 per cent of the hydrolytic activity of a homogenate to this substrate is due to the cholinesterase. Since phenylbutyrate probably does not protect the cholinesterase very well, the protection of the aliesterase may be still better than would be concluded from Fig. 4. The following experiment confirmed this view.

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Flies were homogenized in phenylbutyrate emulsions containing $1{\cdot}2 \times 10^{-8}$ M diazoxon or paraoxon, and the homogenate transferred to Warburg flasks, so that each flask contained 2·2 ml of the reaction mixture with 0·5 flies and a final concentration of 6×10^{-9} M of the inhibitor. The hydrolytic activity was compared with that of controls obtained in a corresponding way but without inhibitor. As eserine eliminates all cholinesterase activity, each measurement was performed with and without eserine. Table 3 shows the results.

TABLE 3 INHIBITION OF ALIESTERASE BY PARAOXON AND DIAZOXON

(Substrate and inhibitor simultaneously added)

	Percentage inhibition by			
	Paraoxon		Diazoxon	
Eserine	−	+	−	+
After 30 min	17	11	23	13
After 60 min	21	17	29	19
After 120 min	27	25	37	29

The inhibitor and the substrate were both present at the time of homogenization and it may thus be assumed that substrate and inhibitor get the same chance of reacting with the enzyme as they do in the *in vivo* inhibition measurements.

From the experimental results reported in this section it can be concluded that the addition of a suitable substrate at the time of homogenization is a simple method for obtaining reliable data on *in vivo* inhibition of the cholinesterase and the aliesterase. Since protection of the enzyme by its substrate is the essential feature of this method it will be referred to as the "protective method".

3. *Results obtained with the "protective method"*

The method finally adopted may be briefly described:

(a) *Inhibition*, in vivo, *at the time of knock-down*. If exposed to the insecticides studied each individual fly passes through a stage of hyperactivity which is followed by a state of prostration (knock-down). As soon as a fly reached that state of paralysis it was removed from the jar in which exposure took place (using a funnel–trap-door system) to a test-tube standing in a mixture of ether and solid carbon dioxide, in which it was kept till further treatment. As soon as all flies were collected in this way they were homogenized. One millilitre of the homogenate obtained was then quickly transferred to a Warburg flask containing 1·2 ml of additional fluid, so that the reaction took place in a final volume of 2·2 ml. Flies for cholinesterase measurement were homogenized in 3% NaCl containing 0·025 M bicarbonate and 0·033 M acetylcholine. The additional fluid contained 3% NaCl and 0·025 M bicarbonate. Each flask contained 1·5 homogenized flies. In the case of aliesterase determinations homogenization took place in a phenylbutyrate emulsion in 1% NaCl and the additional fluid contained 1% NaCl, 0·046 M $NaHCO_3$, and 10^{-5} M eserine. In these experiments each flask contained 0·5 homogenized flies.

The activities measured (μl. CO_2 evolved in the first 30 min) were compared with those obtained in control experiments with flies that were not exposed to insecticides but otherwise treated in exactly the same way. The percentage inhibition could then be calculated.

(b) *Inhibition*, in vivo, *at other stages of poisoning*. Groups of flies were exposed to the insecticides for a certain period and the development of poisoning symptoms noted. At the end of the exposure period the flies were narcotized by CO_2-gas and then homogenized in media containing acetylcholine or phenylbutyrate. Further treatment was similar to that described above.

Table 4 shows the data on *in vivo* inhibition at the time of knock-down. It is clear that cholinesterase inhibition, as measured for whole flies, was low. On the contrary aliesterase inhibition approached 100 per cent in several cases and was always distinctly higher than cholinesterase inhibition.

It seemed useful to investigate what relation exists between the time course of *in vivo* inhibition and the time course of the poisoning. In a previous study[14] we

TABLE 4. INHIBITION *in vivo* AT THE TIME OF KNOCK-DOWN

	Cholinesterase* (%)	Aliesterase† (%)
DDVP	27 (6)	76 (5)
Paraoxon	51 (6)	95 (2)
Parathion	49 (6)	95 (1)
Diazoxon	—	91 (1)
Diazinon	32 (3)	90 (3)
Co-ral	44 (5)	75 (3)

Mean values are given; number of experiments in brackets.

*Protection by acetylcholine 0·015 M
†Protection by phenylbutyrate-emulsion

reported on low *in vivo* inhibition values for the cholinesterase activity in the earlier phases of poisoning by parathion and DDVP. This is in accordance with the low inhibition values at the time of knock-down. Experiments on aliesterase inhibition with DDVP, diazinon and paraoxon showed that soon after exposure to the insecticide the inhibition rises sharply.

Experiments with DDVP. Flies were exposed to DDVP vapour. At the time of knock-down the *in vivo* aliesterase inhibition amounted to about 80 per cent. In an experiment where the first symptoms (hyperactivity) occurred after 38 min it was found that after 10 min the *in vivo* inhibition was 54 per cent. If in this case phenylbutyrate was added after the homogenate had been incubated for 1·5 hr at 27 °C, the inhibition was 75 per cent. The increase from 54 per cent to 75 per cent by incubation in the absence of substrate proves the presence of a small amount of free inhibitor in this case. In another experiment flies were exposed to DDVP vapour for 15 min. At the end of the exposure period the first symptoms were observed. The flies were then divided into three groups of ten flies each, which were further kept in clean jars, where the intoxication progressed. The inhibition value immediately at the end of the exposure period was 84 per cent; after 30 and 120 min values of 87 per cent and 87 per cent were measured; almost complete knock-down occurred in the latter two groups soon after they had been transferred to clean jars. Little or no recovery was observed, neither towards normal behaviour nor towards normal aliesterase activity. In a third experiment flies were exposed for 15 min to a smaller amount of DDVP vapour. No symptoms were observed. At the end of the exposure period the flies were divided into five groups of ten flies each, which were kept in clean jars. These groups were used for *in vivo*-inhibition measurement after 0, 30, 60, 90 and 120 min. The inhibition values found were 35, 23, 44, 35 and 51 per cent, respectively. Although there is a great deal of variation it may be concluded that there is little or no recovery of the aliesterase activity.

Experiments with diazinon. Flies were exposed to diazinon vapour. At the time of knock-down the *in vivo* aliesterase inhibition amounted to about 90 per cent. In an experiment where symptoms occurred after 45 min and 50 per cent knock-down after about 60 min, the *in vivo* inhibition was 28, 90, 92 and 87 per cent after 30, 60, 90 and 120 min, respectively. In a second experiment under similar conditions the *in vivo* inhibition after 30 min exposure amounted to 66 per cent. If the homogenate was incubated before adding substrate the inhibition was 81 per cent. This again points to the occurrence of a small amount of free inhibitor. In a third experiment the flies were withdrawn from the diazinon vapour after 30 min and transferred to clean jars. The inhibition found after 0, 30, 60, 90 and 120 min was 29, 31, 45, 44 and 60 per cent, respectively. The tendency to increase may be explained by the progressive conversion of diazinon into diazoxon. A possible recovery could have been masked by this process.

Experiments with paraoxon. Flies were exposed to a residue of paraoxon on glass. At the time of knock-down *in vivo* inhibition was about 90 per cent. In a first experiment four groups of ten flies each were exposed to paraoxon for 5, 15, 30 and 60 min. The first visible symptoms occurred after 30 min, 50 per cent knock-down after about 60 min. The *in vivo* inhibition values were 63, 66, 83 and 96 per cent, respectively. In a second experiment flies were exposed for 5 min only, to a smaller residue. A control group was continually exposed and showed symptoms of intoxication after 40 min and 50 per cent knock-down after about 3 hr. After 5 min exposure the *in vivo* inhibition amounted to 54 per cent. If phenylbutyrate was added *immediately after* homogenization an inhibition value of 71 per cent was found and if the addition of substrate occurred after 0·5 hr of incubation the inhibition found was 94 per cent. This proves the presence of a large amount of free paraoxon on and/or in the flies at the time of homogenization. This conclusion was confirmed in several other experiments, in which the homogenates were diluted with homogenates of unexposed flies. After 5 min exposure the amount of free paraoxon was approximately 7 ng per fly. It was found that after 30 min exposure the amount of free paraoxon did certainly not exceed this amount. This has not been further investigated.

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DISCUSSION

In a previous paper[13] experiments on the *in vivo* inhibition of esterases by DDVP have been described and discussed. The present paper deals with further investigations into the same subject, in which other poisons have also been used. The results obtained in these investigations confirm the observations previously made that at the time of knock-down by organophosphates the cholinesterase activity in the housefly is only little inhibited. This is especially surprising in view of the general belief that the cholinesterase is present in great excess[19] and that cholinesterase inhibition will only interfere with the normal function of the nervous system if the percentage inhibition approaches 100%. This discrepancy may be explained in one of several ways.

(1) It is possible that the data in the literature on the excess of cholinesterase are incorrect. These data are all based on *in vivo* inhibition experiments in which the enzyme is not protected against free inhibitor, and could, therefore, be too high.

(2) Although the overall inhibition values found in our experiments are low, high percentage inhibition at some essential part of the nervous system cannot be excluded. Actually it has been found that the inhibition of the cholinesterase in the thoraxes of poisoned flies is somewhat higher than that found for whole flies.[14]

(3) It could be that cholinesterase inhibition is less important for the mechanism of intoxication than it is generally thought to be.

Recently Mengle and Casida[20] studied the *in vivo* cholinesterase inhibition of brain cholinesterase after treatment of houseflies with a number of organophosphorus compounds. The occurrence of paralytic symptoms proved to be associated with high inhibition of the enzyme, i.e. 80 per cent inhibition or more in most cases. We are unable to explain the striking difference between these and our own results.

As the organophosphates are also potent inhibitors of the aliesterase present in houseflies it seemed useful to study the *in vivo* inhibition of this enzyme as well. The "protective method" using phenylbutyrate as a substrate made it possible to obtain reliable data for this enzyme. At the time of knock-down invariably high *in vivo* inhibition was observed. It has been suggested[13] that organophosphates might exert their toxic action by the inhibition of the aliesterase. This hypothesis is strongly invalidated by the observation that flies which are resistant to organophosphates all show a very low aliesterase activity.[21] Moreover, nothing is known about the natural substrate and the normal function of the aliesterase present in organophosphate susceptible houseflies, and any theory that ascribes the toxic action of these compounds to aliesterase inhibition must, therefore, be highly speculative.

If an organophosphate is applied to the living insect, inhibition of both the cholinesterase and the aliesterase will occur and the degree of inhibition will depend on the total amount of the inhibitor picked up or formed, its distribution and the rate of reaction with the two enzymes. The cholinesterase and the aliesterase will compete for the organophosphate present, and it may be expected that the presence of the aliesterase will lower the amount of free inhibitor and thus protect the cholinesterase from inhibition. It seems difficult to reconcile this view with the low aliesterase level in organophosphate resistant flies. Recent work, however, has shown that, at least in some strains, resistance is probably due to a change in the nature of the enzyme protein, which results in a loss of aliesterase activity but enhances the capacity of eliminating (probably hydrolysing) organophosphates.

The data presented in this paper clearly show that the inhibition of the aliesterase precedes that of the cholinesterase in the living insect. Corresponding investigations on the reaction of organophosphates with the two enzymes in homogenates will be published shortly. If the results obtained in these *in vitro* experiments are compared with those of the present paper, it appears likely that structural barriers play an important role in the living fly, the aliesterase being more accessible to the inhibitor than the cholinesterase.

The experimental results on *in vivo* inhibition in earlier stages of intoxication and after withdrawal of the flies from the toxicants indicate that aliesterase inhibition is not readily reversible *in vivo*.

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CHOLINESTERASE INHIBITION BY GALANTHAMINE AND LYCORAMINE

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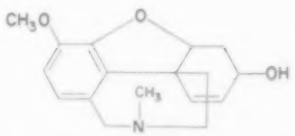
Abstract—Galanthamine, a recently isolated alkaloid, has been found to inhibit the acetylcholinesterase of muscle and the cholinesterase of plasma and brain. The inhibition of the muscle enzyme by galanthamine is greater than that produced by pyridostigmin, a compound of known usefulness, but less than that of neostigmin. The muscle cholinesterase inhibition produced by lycoramine was found to be about equal to that of pyridostigmin. These alkaloids are thus of interest because of the possibility of their therapeutic usefulness.

GALANTHAMINE and lycoramine are alkaloids which have recently been isolated from plant bulbs of the Amaryllidaceae family.[1–4] Galanthamine is an amine believed to have the chemical structure:



A slightly different structure has been assigned to galanthamine by Proskurnina and Yakovleva.[5] The spectral analysis of samples from different sources appears to be identical. Galanthamine is referred to as lycoremine by Uyeo and Kobayashi.[2] Lycoramine is the dihydro-derivative of galanthamine and contains no double bond in the hydroxyl-substituted ring. These alkaloids structurally resemble morphine, which is known to influence cholinesterase activity, since it can antagonize the inhibition of plasma cholinesterase by prostigmine and inhibit the esterases of plasma and red blood cells.[6, 7] Thus, since galanthamine has been reported to inhibit cholinesterase, to be of benefit in the treatment of myasthenia gravis,[7] and to increase the sensitivity of muscle to acetylcholine,[8] we have determined the cholinesterase activity of a muscle homogenate in the presence of each compound, and compared the inhibition to that produced by pyridostigmin and neostigmin, compounds with known activity and usefulness. Fig. 1 shows that galanthamine hydrochloride inhibits muscle cholinesterase more than pyridostigmin bromide, but less than neostigmin bromide. Lycoramine hydrobromide produced about the same amount of inhibition as pyridostigmin. Galanthamine hydrochloride was also found to inhibit the cholinesterase of dog plasma (Fig. 1) and the cholinesterase of cat brain; the brain enzyme was inhibited 50 per cent at a 2×10^{-5} M concentration.

We used a muscle homogenate prepared from the entire hind limb musculature of a dog and all the curves plotted from the activity of muscle enzymes were obtained with

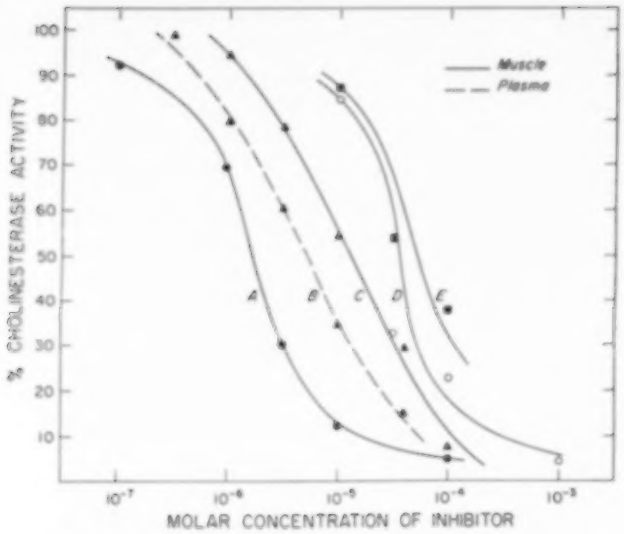


FIG. 1. Inhibition of muscle and plasma cholinesterase.
A. Neostigmin Br. B. Galanthamine HCl (plasma).
C. Galanthamine HCl. D. Pyridostigmin Br. E. Lycoramine HBr.

the same batch of homogenate. The muscles were homogenized in and washed three times with saline and then diluted 1 : 5 with a bicarbonate buffer with a final molar concentration of $NaHCO_3$ 0·025, NaCl 0·15, $MgCl_2$ 0·04, and equilibrated with 95% N_2 and 5% CO_2. The activity of 0·92 g (wet weight) of muscle was measured at 10 min intervals for 30 min in the Warburg apparatus using a 0·01 M acetylcholine chloride substrate. Twelve flasks were used to determine each point on the curves depicting muscle activity, including corrections for pressure, heat-inactivated tissue, and spontaneous hydrolysis of the substrate. The inhibitor and the substrate were tipped into the homogenate simultaneously from different side arms and incubated with shaking for 10 min prior to beginning measurement of enzyme activity. Homogenates prepared and tested in this way hydrolyse acetyl-β-methylcholine, show only slight activity in splitting butyrylcholine or benzoylcholine and are inhibited by an excess of an acetylcholine substrate. The dog muscle enzyme which these alkaloids inhibit can thus be considered chiefly acetylcholinesterases.

Acknowledgement—The alkaloids used in these experiments were isolated from the bulbs of *Narcissus pseudo-narcissus* L. (King Alfred variety) by Dr. W. Wildman of the Laboratory of Chemistry of Natural Products of the National Heart Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

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INHIBITORS OF AROMATIC ESTERASE OF HUMAN SERUM

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Abstract—Thirty-six drugs in a concentration of 10^{-4} were tested for influences on aromatic esterase activity. About one-third of them caused some inhibition. The three strongest inhibitors, in increasing order, were atropine, naphazoline, and chlorpromazine. The inhibition constant, K_i, of chlorpromazine was 3×10^{-5}. Twelve further phenothiazine derivatives showed an inhibitory potency of a similar magnitude, i.e., the inhibitory effect was nearly independent of molecular substitutions. Calcium did not influence the inhibition. Most inhibitors of aromatic esterase are known to be capable of influencing to various degrees other enzymes.

INTRODUCTION

THE name aromatic esterase was introduced by Mounter and Whittaker[1] because this enzyme hydrolysed esters of phenol and substituted phenols; this means that the enzyme is capable of combining with these agents. Since many drugs contain a phenol moiety we wondered whether these drugs also act as competitive inhibitors of aromatic esterase. Hence, the drugs first checked were related to phenol, but in the course of this investigation we came to include aromatic drugs of divergent structures.

A-esterase is a synonym for aromatic esterase.[2] It occurs in the albumin fraction of human serum.[3, 4] Resistance to inhibition by eserine and organophosphorus compounds is characteristic of this esterase.[1,2] Aldridge[2] found that the A-esterase not only is insensitive to organophosphorus compounds, but also it can hydrolyse at least one of them, diethyl *p*-nitrophenyl phosphate. Augustinsson and Olsson[5] selected eight compounds frequently employed as enzyme inhibitors and reported that the aromatic esterase from the plasma of some swine was resistant to all inhibitors tested. In a previous study[4] on separation of human A-esterase and pseudocholinesterase by electrophoresis, we found that barbital in a concentration of 8×10^{-3} M caused 50 per cent inhibition, while ethylenediaminetetra-acetic acid (EDTA; Versene) in a concentration of 5×10^{-5} completely blocked the activity of A-esterase. The activity blocked by EDTA could be restored with the aid of different bivalent metal ions.

EXPERIMENTAL

Assay of esterase activity

The Beckman Recording Spectrophotometer model DK2 was used for the determination of the activity of aromatic esterase. The activity was measured by recording the initial change of absorbance at 269 mμ which occurred during hydrolysis

* This work forms part of a thesis submitted by A. V. Marton for the degree of Master of Arts in Pharmacology, University of Toronto, 1959.

of phenyl acetate.[4, 6] The reaction was conducted in absorption cells with a light path of 1 cm at a temperature of 25·6 ± 0·3°C. The duration of a single measurement was 2 min.

Hydrogen-ion concentrations of the reaction mixtures were determined with a glass electrode in a Beckman pH-meter (model G) before and after each measurement; these values did not decrease more than 0·02 pH unit during the reaction. The solvent was always a mixture of equal parts of phosphate buffer (0·067 M, pH 7·4) and of tris-(hydroxymethyl)-amino-methane buffer (0·1 M, pH 7·4). This mixture of buffers was also used as an optical blank.

All measurements were carried out in quadruplicate. The average values of initial slopes were recorded.

Esterase

The sources of enzyme were samples of human serum which were free of haemolysed erythrocytes. The final dilution of crude serum was 1 : 400. Under these conditions the hydrolysis of phenyl acetate depends on the aromatic esterase activity but not on that of cholinesterase; the hydrolysis rate in crude serum was found to be proportional to that in the albumin fraction of the same sample of serum.[4]

Substrate

The substrate was a freshly prepared solution of phenyl acetate. Its initial concentration in the absorption cell was $3{\cdot}9 \times 10^{-4}$ M.

Esterase inhibitors

Forty-eight drugs were used as possible inhibitors. All the materials tested were compounds intended for medical use or commercially available products of equivalent purity. Stock solutions of all these compounds were prepared in a concentration of 4×10^{-4} M; the concentration in contact with the enzyme in the absorption cell was 1×10^{-4} M. The drugs were dissolved in buffer shortly before use and added simultaneously with the substrate to the serum.

RESULTS

Tables 1 and 2 show the results of the experiments with a standard concentration of 0·1 mM of various drugs. Fourteen of the forty-eight drugs examined did not have any influence on the rate of hydrolysis of phenyl acetate; eight of them caused a slight inhibition. The remaining twenty-six drugs inhibited the A-esterase in a range from 14 to 100 per cent.

All the barbiturates investigated, as well as thimerosal and methamphetamine, inhibited the hydrolysis of phenyl acetate between 10 and 20 per cent. Atropine, homatropine, neophenylephrine, dibucaine, papaverine and yohimbin caused a block of from 20 to 40 per cent. Fifty per cent or more inhibition was found with all the phenothiazine derivatives and with naphazoline, while EDTA completely blocked the enzymic hydrolysis.

The blocking effects of chlorpromazine and of naphazoline were investigated in greater detail. Experiments were carried out with these compounds in order to determine the dissociation constant of the enzyme–inhibitor complex, or in other words the

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TABLE 1. EFFFCT OF DRUGS ON THE ACTIVITY OF AROMATIC ESTERASE

Compound	Synonyms	Percentage of original enzyme activity
Acetylsalicylic acid	Aspirin	>90
Amphetamine sulphate	1-Phenyl-2-aminopropane; benzedrine	100
Atropine sulphate	DL-Hyoscyamine	64
Azacyclonol HCl	α-(4-Piperidyl) benzhydrol; frenquel	85
Barbital Na	Barbitone; 5 : 5-diethylbarbituric acid	>90
Benzocaine HCl	Ethylamino-benzoate	100
Caffeine Na-benzoate	1 : 3 : 7-trimethylxanthine	100
Chlorpromazine HCl	Largactil; thorazine	31
Cocaine HCl	Methylbenzoyl ecgonine	100
Decamethonium bromide	Syncurine; C_{10}	>90
Dibucaine HCl	Cinchocaine; nupercaine HCl	75
EDTA	Ethylenediaminetetra-acetic acid; versene	0
Ephedrine sulphate		>90
Histamine phosphate		100
Homatropine HBr	Tropin mandelic ester	79
Iproniazid phosphate	*iso*Propyl isoniazid; marsilid phosphate	100
Mephenesin	Myanesin; lissephen; tolserol	100
Meprobamate	Miltown; equanil	>90
Methamphetamine HCl	Methedrine	86
Naphazoline HCl	α-Naphthyl-2-methylimidazoline; privine	49
Naphthylethylenediamine		100
Niamide	*N-iso*Nicotinoyl-N^1-(-*N*-benzylcarboxamido-ethyl)-hydrazine	100
DL-Norepinephrine HCl	Noradrenaline; arterenol; levophed	100
L-Norepinephrine bitartrate	L-Noradrenaline	100
Papaverine HCl		73
Pentylenetetrazol	Leptazol; cardiazol; metrazol	100
Phenylephrine HCl	Neosynephrine	75
Pilocarpine HCl		100
Procaine HCl	Novocaine	>90
Quinine HCl		>90
Secobarbital sodium	Seconal	83
Tetramethylammonium bromide	TMA	>90
Thimerosal	Merthiolate sodium	83
Thiopental sodium	Pentothal	82
Urethane	Ethyl urethane	100
Yohimbine HCl		69

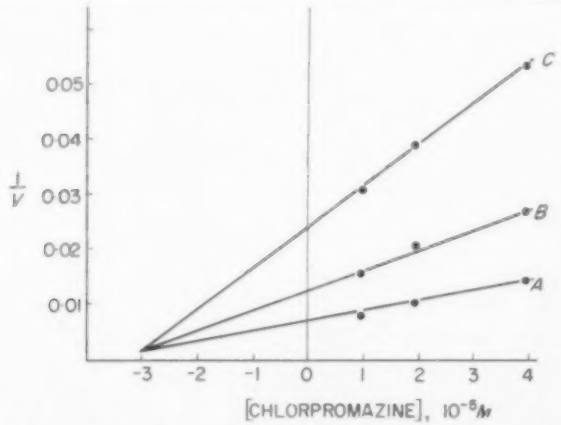
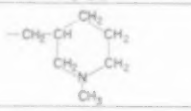
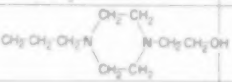
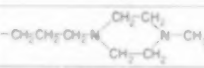
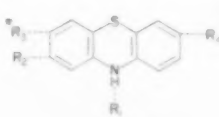


FIG. 1. Effects of chlorpromazine on aromatic esterase activity. The abscissae indicate molar concentrations of inhibitor, the ordinates give reciprocals of the hydrolysis rates of phenylacetate in arbitrary units. The concentration of phenylacetate was: $3{\cdot}7 \times 10^{-4}$ M for the data on line *A*; $1{\cdot}85 \times 10^{-4}$ M for the data on line *B*; $9{\cdot}25 \times 10^{-5}$ M for the data on line *C*.

inhibitor constant, K_i. The determination of K_i required measurements of initial velocities with a number of different concentrations of inhibitor and of substrate. The reciprocal of the velocity of the enzyme reaction ($1/V$) was plotted against the concentration of inhibitor (i), as described by Dixon.[7, 8] The results are shown in Fig. 1.

TABLE 2. EFFECT OF PHENOTHIAZINE DERIVATIVES ON THE ACTIVITY OF AROMATIC ESTERASE

Inhibitor	Synonym	Substituents of the Phenothiazine Nucleus* R_1	R_2	R_3	R_4	Percent of original Enzyme activity
Chlorpromazine	Largactil	$-CH_2-CH_2-CH_2-N(CH_3)_2$	CL	H	H	31
Diethyzine HCL	Diparcol	$-CH_2-CH_2-N(C_2H_5)_2$	H	H	H	34
Ethopropazine HCL	Parsitan	$-CH_2-CH(CH_3)-N(C_2H_5)_2$	H	H	H	43
Mepazine	Pacatal		H	H	H	29
Methylene blue**	Methylthionine	H	H	$N(CH_3)_2$	$N(CH_3)_2$	31
Levomepromazine	Nozinan	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	OCH_3	H	H	25
Perphenazine	Trilafon		CL	H	H	48
Phenothiazine HCL		H	H	H	H	39
Proclorperazine	Stemetil		H	CL	H	24
Promethazine	Phenergan	$-CH_2-CH(CH_3)-N(CH_3)_2$	H	H	H	32
Tetramepromazine	Lispamol	$-CH_2-CH(N(CH_3)_2)-CH_2-N(CH_3)_2$	H	H	H	27
Triflupromazine	Vesprin	$-CH_2-CH_2-CH_2-N(CH_3)_2$	$C(F)_3$	H	H	43
Trimeprazine	Panectyl Temaril	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	H	H	H	33



* R_1

** The formula given here corresponds to the Leuco form of Methylene blue, though it was used in its oxidized form.

The K_i values obtained for chlorpromazine and naphazoline were $3{\cdot}15 \times 10^{-5}$ M and $6{\cdot}3 \times 10^{-5}$ M, respectively. Using the method of Hunter and Downs[9] for the evaluation of the experimental data the K_i values were: 4×10^{-5} M for chlorpromazine and $6{\cdot}9 \times 10^{-5}$ for naphazoline.

Five bivalent cations (Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}) at a concentration of $2{\cdot}5 \times 10^{-4}$ M were capable of restoring the A-esterase activity which had been blocked completely by 10^{-4} M EDTA.[4] When 50 per cent of the original activity of A-esterase had been blocked by phenothiazine derivatives, no restoration could be achieved with the aid of $2{\cdot}5 \times 10^{-4}$ M Ca^{2+}.

DISCUSSION

In their studies on A-esterase of swine plasma, Augustinsson and Olsson[5] used the following inhibitors: physostigmine salicylate, tetra*iso*propylpyrophosphoramide, bis-mono*iso*propylaminofluorophosphine oxide (mipafox), 10-(1-diethylamino-propionyl)-phenothiazine hydrochloride (Astra 1397), 1:5-bis-(4-trimethylammonium phenyl)-pentane-3-one-di-iodide (62 C 47), bis-(piperidino methylcoumaranyl-5)-ketone (3318 CT), sodium arsanilate (Atoxyl) and quinine sulphate. They found that the enzyme was inhibited by none of these compounds. We have not tested Astra 1397, but judging from our results with other phenothiazine derivatives, it would probably have caused inhibition under our experimental conditions. We also found quinine to have some blocking effect. At present, one cannot decide whether these deviations from the results of Augustinsson and Olsson are due to a difference of species or methods. While we have not reported on physostigmine or organophosphorus inhibitors in this study, we have previously tested these inhibitors[1] with the same result as Augustinsson and Olsson.

In the experiments reported here, thirteen phenothiazines were investigated with different substituents on either the 10- or the 2-position or both, of halogenated and halogen-free derivatives. All these compounds showed a blocking effect of the same magnitude on aromatic esterase of human serum. Since the inhibitory effect of these compounds does not seem to be dependent on the substituent of the molecule, it is probably a property of the phenothiazine nucleus itself.

The most potent inhibitor of the tested drugs was EDTA, which has a fairly simple aliphatic structure. All other drugs which had a significant inhibitory effect on A-esterase of human serum were compounds of a more complicated chemical structure containing some kind of ring system in the molecule. EDTA acts as a chelating agent so that its inhibitory effects can be overcome by an excess of Ca^{2+} and some other cations; the inhibitory effect of the phenothiazine is refractory to calcium so that these agents act by a different mechanism.

Using graphical methods[7, 8] for the determination of the inhibitor constant, K_i, by plotting $1/V$ against i, a straight line is obtained; v represents the reaction velocity in the presence of a concentration, i, of the inhibitor at a given concentration of substrate. With another substrate concentration, a second straight line is obtained which intersects the first line. At this point of intersection one can obtain the value of K_i. This value lies above the base-line in case of competitive inhibition, and lies on the base-line in case of a non-competitive inhibitor. In the present work K_i values of chlorpromazine and of naphazoline were determined using this method. One cannot judge with certainty whether the inhibition is competitive or non-competitive: the intersection of the straight lines is close to the base-line, but the poor water solubility of phenyl acetate made it impossible to use the substrate in a concentration high enough to saturate the enzyme and to approach the maximum velocity (V_{max}). For the same reason it was not possible to make a reliable estimate of the Michaelis constant for phenyl acetate and aromatic esterase, either directly or through the K_i determinations.

No physiological function of A-esterase is known, and, with the exception of EDTA, no drug stands out as a particularly potent inhibitor of aromatic esterase. Relatively strong inhibiting drugs were atropine, naphazoline, and chlorpromazine; it is difficult to think of a common effect of atropine and naphazoline which could be

attributed to aromatic esterase inhibition. One may note that atropine,[10, 11] naphazoline,[12] and chlorpromazine[11, 13] are all capable of interacting with other enzymes. All these drugs can block both true and pseudocholinesterase; of the latter enzyme, both the usual form and the atypical variety[14] can be inhibited. In some rabbit sera, atropine is hydrolysed by atropine esterase.[15] Naphazoline has been shown to inhibit both mono- and di-aminoxidase.[12] Hence, there are several enzyme proteins known with which these drugs can combine *in vitro*, and most likely there are numerous further proteins. One feels compelled to assume that each drug *in vivo* occupies different proteins; the relative affinities characteristic for a drug and any given protein may then be said to determine its *receptor spectrum*.

After the manuscript of the present paper was completed, we noted a recent article by Erdös *et al.*[16] in which trivalent rare earth cations were described as the strongest inhibitors of aromatic esterase to-date.

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SYNTHESIS AND PHARMACOLOGY OF NICOTINYLCHOLINE AND THREE BISQUATERNARY RELATED DERIVATIVES

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Abstract—Nicotinylcholine and three related bisquaternary derivatives, nicotinylcholine iodide methiodide, nicotinylhomocholine iodide methiodide and pyridyl-3-acetycholine iodide methiodide, have been synthesized and studied pharmacologically. Nicotinylcholine is in general less potent than naturally-occurring choline esters. It is practically devoid of muscarinic actions, but in cats in doses of 5–10 mg/kg body weight it possesses pronounced nicotinic actions (ganglion-stimulating and neuromuscular-blocking actions). It is split by butyrocholinesterase at approximately the same rate as acetylcholine. On the frog rectus nicotinylcholine is about 300 times less potent than acetylcholine.

The corresponding bisquaternary compound, nicotinylcholine iodide methiodide, is not split by cholinesterases, and in doses of 10–20 mg/kg body weight it possesses ganglion-blocking properties. It has no effect on neuromuscular transmission as revealed by the cat sciatic nerve-gastrocnemius preparation. The other two bisquaternary compounds, nicotinylhomocholine iodide methiodide and pyridyl 3-acetylcholine iodide methiodide, have neuromuscular blocking actions in doses of 5–10 mg/kg body weight, but have little effect on the cat blood pressure. They are not split by cholinesterases.

ALIPHATIC choline esters have been studied in great detail for many years.[1, 2] The growing number of naturally-occurring esters of this type, both saturated[3, 4] and unsaturated,[5, 6] have prompted the study of numerous synthetic analogues. Among the naturally-occurring choline esters, so far only one compound containing a cyclic component has been found, urocanylcholine (murexine).[7]

In spite of the fact that nicotine has played such an important role in the study of the nervous system only scanty data are available concerning the choline ester of nicotinic acid, nicotinylcholine.[8] This paper deals with the synthesis and pharmacological effects of nicotinylcholine and some quaternary analogues, since the quaternization at the heterocyclic nitrogen might be expected to result in drugs with interesting pharmacological properties.

EXPERIMENTAL

Preparation of the esters

Nicotinylcholine iodide. A solution of 0·31 mole of 2-dimethylaminoethanol in 50 ml of benzene was added to 0·10 mole of crude nicotinyl chloride in 75 ml of benzene. The latter reagent was obtained from potassium nicotinate and oxalyl

chloride according to Wingfield *et al.*[9] The reaction mixture was refluxed for 4 hr. The dimethylaminoethanol hydrochloride formed was separated by filtration, and the filtrate was evaporated *in vacuo*. A viscous oil was obtained which was washed with 10 ml of cold water, dissolved in ether and dried with anhydrous sodium sulphate. After removal of the ether, 2-dimethylaminoethyl nicotinate was distilled, b.p. 90 °C/0·10 mm Hg. Yield 40 per cent, calculated on nicotinic acid.

2-Dimethylaminoethyl nicotinate (0·10 mole) was dissolved in 130 ml of anhydrous ether and 0·10 mole of methyl iodide was added. The reaction mixture was kept overnight at room temperature and yellow crystals of nicotinylcholine iodide were filtered off, washed with anhydrous ether and recrystallized from absolute ethanol. That quaternization of the ester had taken place at the nitrogen of the ester group and not at the pyridine nitrogen was shown by the isolation of choline as the reineckate after hydrolysis, as follows.

The quaternized ester (0·1017 g) was dissolved in 50 ml of water; 20 ml of this solution were hydrolysed by means of 20 ml of a 10% trisodium phosphate solution. To the hydrolysate 10 ml of a freshly-prepared 2% reineckate solution were added, and after 2 hr the precipitate was filtered off, washed with 10 ml of cold water and dried to constant weight at 110 °C. Found: 0·0510 g. Calc. for choline reineckate: 0·0511 g. Choline chloride (0·1000 g) treated in the same manner gave 0·1217 g choline reineckate against calculated 0·1211 g, while the hydrolysed methiodide of m ethy nicotinate gave no precipitation with the reineckate solution of the pH in question.

Nicotinylcholine iodide methiodide. 2-Dimethylaminoethyl nicotinate (0·10 mole) was dissolved in 200 ml of absolute methanol and 0·21 mole of methyl iodide was added. After 5 days the yellow crystals were filtered off and washed with anhydrous ether.

Nicotinylhomocholine iodide methiodide. 3-Dimethylaminopropyl nicotinate was prepared from nicotinyl chloride and 3-dimethylaminopropanol[10] in the same way as the ethyl ester: b.p. 95·5–97 °C/0·10 mm Hg, yield 61 per cent. The ester (0·10 mole) was dissolved in 200 ml of absolute ethanol, and the solution was refluxed for 3 hr with 0·21 mole of methyl iodide. After 6 days the yellow crystals were filtered off and washed with warm ethanol.

Pyridyl-3-acetylcholine iodide methiodide. 2-Dimethylaminoethyl pyridyl-3-acetate was synthesized in the same way as the corresponding nicotinate from pyridyl-3-acetic acid[11] and 2-diemethylaminoethanol. The viscous oil obtained was not purified by distillation, because it showed a tendency to decompose. The yield of the undistilled ester was 62 per cent. The crude ester (0·10 mole) dissolved in 400 ml of absolute ethanol was refluxed for 3 hr with 0·22 mole of methyl iodide and the crystals formed after 6 days were filtered off and washed with warm ethanol.

The formulae and analytical data of the compounds synthesized are given in Table 1.

Pharmacological and biochemical methods

Recording of respiration, blood pressure and muscular contractions. The effects of the esters were studied in ten anaesthetized cats. Anaesthesia was induced with ether followed by allobarbitone (50 mg/kg body weight) and was maintained by further

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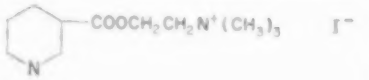
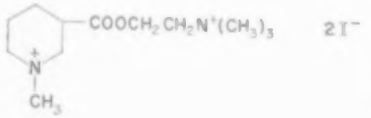
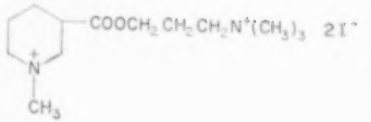
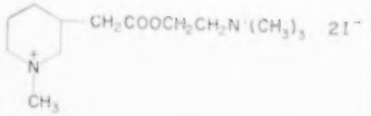
TABLE I. ANALYTICAL DATA

Compound	Formula	Molecular weight	% C Calc.	% C Found	% H Calc.	% H Found	% I Calc.	% I Found	Yield in % calculated on the acid
Nicotinylcholine iodide (L 7)		336·2	39·3	39·1	5·1	5·0	37·7	37·7	34
Nicotinylcholine iodide methiodide (L 3)		478·1	30·1	30·2	4·2	4·2	53·1	52·7	20
Nicotinylhomocholine iodide methiodide (L 42)		492·2	31·7	31·5	4·5	4·3	51·6	51·8	37
Pyridyl-3-acetylcholine iodide methiodide (L 30)		492·2	31·7	32·3	4·5	4·3	51·6	49·0	14

small doses of allobarbitone. Drugs were injected intravenously through a plastic cannula in the femoral vein. Respiration, blood pressure, and contractions of the gastrocnemius muscle and of the nictitating membrane were recorded by passing the output of suitably arranged transducers into a Grass Model 5 Polygraph. Respiration was recorded as pressure differences in the tracheal cannula by means of a pressure transducer (Model PT 5, Grass Instrument Corporation). Blood pressure was recorded by means of a Statham electromanometer from the left carotid artery.

The effects of drugs on neuromuscular transmission were determined by recording semi-isometric contractions of the gastrocnemius muscle in response to supramaximal electrical stimulation of the sciatic nerve. A force displacement transducer Model FT 10, Grass Instrument Corporation, was used for recording the muscle contractions and the electrical stimuli were applied to the nerve distally to a crushed region by means of a Grass stimulator, Model S4, and shielded silver electrodes.

The neuromuscular blocking effect of the drugs is expressed in terms of the PD_{50} (namely, the dose required to produce 50 per cent blockade) and of the duration of such a block. To determine these quantities, the drugs were injected intra-arterially through a cannula in the femoral artery of the leg opposite to that being stimulated, the tip of the cannula lying proximally to the aortic bifurcation. The percentage diminution of twitch height and duration of block were recorded for three doses and the PD_{50} and the corresponding duration estimated by graphical interpolation. Succinylcholine has been included for comparison. Contractions of the nictitating membrane were recorded by means of force-displacement transducer FT 03. The cervical sympathetic trunk was exposed and dissected from the vagus. The excitability of the membrane was tested by stimulating the preganglionic trunk for 10–15 sec with supramaximal rectangular pulses of duration of 0·5 msec and frequency of 20 c/s before and after administration of drugs.

Frog rectus abdominis muscle preparations. The procedure was essentially that of Chang and Gaddum.[12] In this and the following preparations the activity of the esters was expressed as the relative molar potency in percentage units with acetylcholine or succinylcholine as standards.

Guinea-pig ileum preparations. A test bath of 6 ml volume was used. An automatic timer regulated the cycle of operations except for the addition of the compounds which was made manually in a small volume on top of the test bath.

Rat phrenic nerve–diaphragm preparation. The procedure was that described by Bülbring.[13] A test bath of 18 ml volume was used. The drug was left in contact with the preparation for 3 min. The nerve was stimulated with supramaximal single shocks, 10 per min of 0·2 msec duration.

Hydrolysis of esters by cholinesterases. This was measured electrometrically at 25 °C and pH 8 in buffer as described by Tammelin.[14] The following preparations were used: purified human plasma cholinesterase stated to correspond to Cohn's plasma fraction IV-6-3 (AB Kabi), human red cell cholinesterase prepared by haemolysing red cells with distilled water. Serum cholinesterase was inactivated by heating for

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30 min at 70 °C, red cell cholinesterase by using a selective inhibitor, 1 : 5-bis (4-*N*-allyl-*N*-methylaminophenyl)-pentanone-3 dimethobromide (B.W. 284C51),[15] at a final concentration of 1×10^{-5} M. The substrate concentrations were: acetylcholine, 25 mM (human plasma cholinesterase) or 8 mM (human red cell cholinesterase), other esters 8 mM. Enzyme concentrations were adjusted to give convenient rates of hydrolysis with the different substrates. The rate of hydrolysis was expressed as μmoles acid liberated per ml per min.

RESULTS

Assay of the new esters on conventional test organs like the frog rectus and the guinea-pig ileum revealed that the nicotinylcholines had a very feeble action in comparison with acetylcholine. The nicotinic action (frog rectus) was, however, considerably stronger than the muscarine-like action (guinea-pig ileum) which on the whole may be said to be lacking (Table 2).

TABLE 2. ACTIVITY OF NICOTINYLCHOLINE AND THREE QUATERNARY ANALOGUES ON FROG RECTUS ABDOMINIS MUSCLE AND GUINEA-PIG ILEUM

Ester	Relative molar potency (acetylcholine = 100)	
	Frog rectus	Guinea-pig ileum
Nicotinylcholine iodide	0·33	<0·002
Pyridyl-3-acetylcholine iodide methiodide	0·14	<0·002
Nicotinylhomocholine iodide methiodide	<0·05	<0·002
Nicotinylcholine iodide methiodide	<0·05	<0·002

Hydrolysis by cholinesterases

All the esters synthesized were tested for their hydrolysis by cholinesterase, which was compared with that of acetylcholine under identical conditions. At the substrate concentrations used the hydrolysis of only one ester, nicotinylcholine iodide, was accelerated by the presence of an enzyme. Purified human serum cholinesterase splits nicotinylcholine iodide at roughly the same rate as acetylcholine iodide is split. By contrast nicotinylcholine iodide is not split at all by human red cell cholinesterase (Table 3).

TABLE 3. HYDROLYSIS OF NICOTINYLCHOLINE IODIDE BY CHOLINESTERASES
(Substrate concentrations in mM given in brackets)

Enzyme preparation	Acid liberated (μmole ml^{-1} min^{-1})	
	Acetylcholine iodide	Nicotinylcholine iodide
Human serum cholinesterase	81×10^{-5} (25)	74×10^{-5} (8)
Human red cell cholinesterase	85×10^{-5} (8)	0 (8)

When the various esters in increasing concentrations were added to the reaction mixture before acetylcholine they showed no appreciable inhibition of the hydrolysis of the latter compound by red cell cholinesterase.

Effect on neuromuscular transmission

When the compounds were tested in two ways on the neuromuscular transmission (cat sciatic nerve gastrocnemius preparation and rat phrenic nerve diaphragm preparation) only three of the esters were found to have an appreciable neuromuscular blocking effect. The relative molar potencies in comparison with succinylcholine are seen in Table 4. On both test organs the compounds showed the same sequence of activity.

TABLE 4. NEUROMUSCULAR BLOCKING ACTIVITY OF NICOTINYLCHOLINE AND TWO QUATERNARY ANALOGUES

Ester	Cat sciatic nerve gastrocnemius preparation				Rat phrenic nerve diaphragm preparation
	PD 50 (mg/kg)	PD 50 (μM/kg)	Duration	Relative molar potency	Relative molar potency
Succinylcholine iodide	0·004	8×10^{-3}	6 min 20 sec	100	100
Pyridyl-3-acetylcholine iodide methiodide	0·29	6×10^{-1}	5 min 20 sec	1·3	5·1
Nicotinylcholine iodide	0·67	2	1 min 10 sec	0·4	1·7
Nicotinylhomocholine iodide methiodide	3·7	7·5	7 min 20 sec	0·1	<0·1

The neuromuscular blocking effect is considerably less than that for the commonly used neuromuscular blocking agents of naturally occurring or synthetic origin. The duration of recovery from a 50 per cent blockade for two of the compounds was of the same order of magnitude as of that for succinylcholine, when tested after intraarterial injection.

Effect on the respiration

From the experiments shown in Figs. 1–4 it is apparent that all the esters studied had negligible effect on the respiration.

Effect on blood pressure

The effects of the esters on blood pressure at doses of 5–10 mg/kg body weight proved to be extremely variable in the series studied. Thus nicotinylhomocholine iodide methiodide showed no appreciable effect on the blood pressure (Fig. 3). Nicotinylcholine iodide constantly showed a biphasic pressor effect (Fig. 1). Pyridyl-3-acetylcholine iodide methiodide exhibited a fleeting depressor response (Fig. 2) whereas nicotinylcholine iodide methiodide caused a depression of considerable duration (Fig. 4).

Effect on the nictitating membrane

The effect of nicotinylcholine iodide and nicotinylcholine iodide methiodide on ganglionic transmission was measured by the height of contraction of the nictitating

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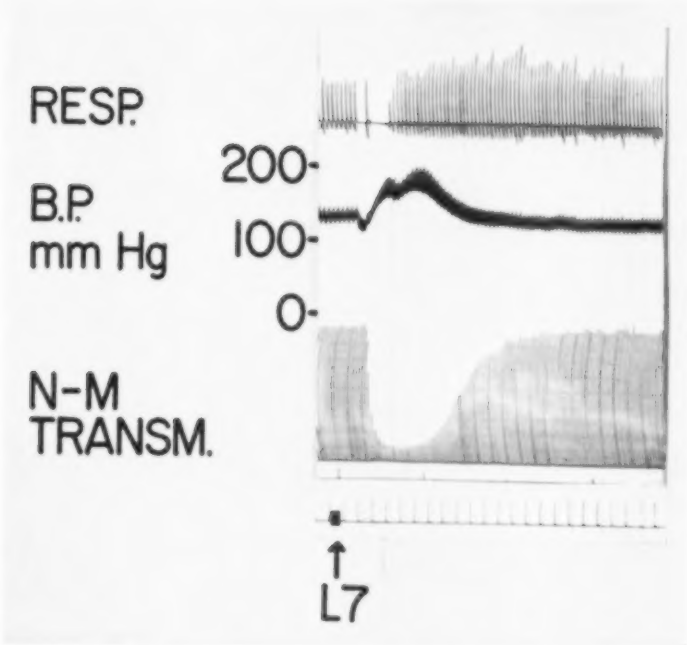

FIG. 1. Effect of nicotinylcholine iodide (L7) on respiration, blood pressure and neuromuscular transmission: 5 mg/kg body weight i.v.; cat 2·1 kg; anaesthetic, allobarbitone; time, 10 sec.

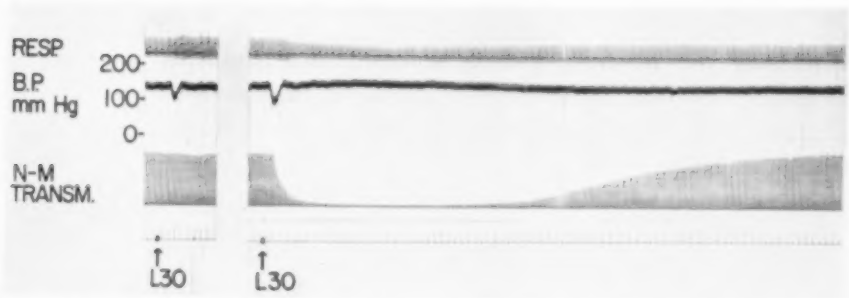

FIG. 2. Effect on pyridyl-3-acetylcholine iodide methiodide (L30) on respiration, blood pressure and neuromuscular transmission: 2·5 resp 5 mg/kg body weight i.v.; cat 2·1 kg; anaesthetic, allobarbitone; time, 10 sec.

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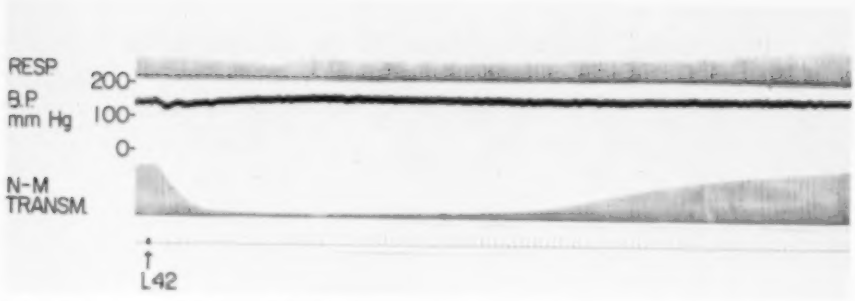

FIG. 3. Effect of nicotinylhomocholine iodide methiodide (L42) on resprespiration, blood pressure and neuromuscular transmission: 5 mg/kg body weight i.v.; cat 2·1 kg; anaesthetic, allobarbitone; time, 10 sec.

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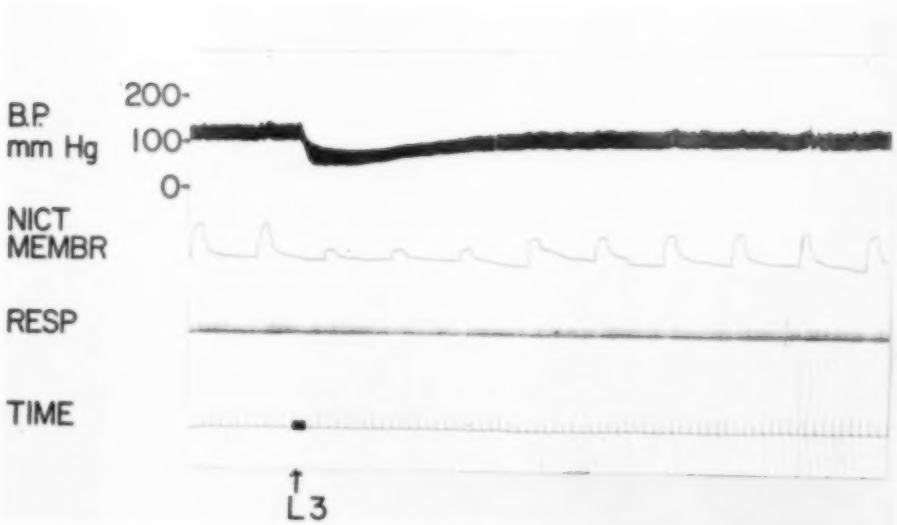

FIG. 4. Effect of nicotinylcholine iodide methiodide (L3) on blood pressure, nictitating membrane and respiration: 20 mg/kg body weight i.v.; cat 2·5 kg; anaesthetic, allobarbitone; time, 10 sec. Intermittent stimulus of isolated central stump of cervical sympathetic trunk 10 sec with rectangular pulses of 0·5 msec duration at a frequency of 20 c/s.

membrane after stimulation of the preganglionic fibres to the superior cervical ganglion at regular time intervals. Nicotinylcholine iodide constantly showed a potentiation of the contractions, whereas nicotinylcholine iodide methiodide decreased the contraction height considerably (Fig. 4). The other esters were inactive on the ganglionic transmission in comparable doses.

DISCUSSION

The synthetic heterocyclic esters in all test organs studied showed much less activity than both murexine[16, 17] and naturally-occurring aliphatic esters.[17, 18] The order of magnitude for a similar response was for the synthetic compounds roughly around 5 mg/kg body weight and for the natural esters 0·1–0·3 mg/kg body weight.

Nicotinylcholine iodide, a compound of considerable theoretical interest as mentioned in the introduction, has the following characteristics: it is easily split by human serum cholinesterase, has a fleeting neuromuscular blocking action, possesses a considerably pressor activity and potentiates the response of the nictitating membrane to preganglionic stimulation. These effects coincide well with the nicotine-like effects of acetylcholine. When nicotinylcholine is converted into the corresponding bisquaternary compound the effect on the blood pressure is transformed into a prolonged depression which coincides with the ganglion blockade (Fig. 4), and is no doubt due to this. Also the effect of nicotinylcholine iodide on the neuromuscular transmission disappears with quaternization. Another feature is that the new compound is no longer hydrolysed by butyrocholinesterase.

The high rate of hydrolysis of nicotinylcholine by butyrocholinesterase is surprising in view of the fact that none of the bisquaternary esters is attacked by butyrocholinesterase but consistent with the well-known high degree of hydrolysis rate of other cyclic choline esters like benzoylcholine previously used as a selective substrate for butyrocholinesterase.[19] The presence of two quaternary groups apparently makes the compounds less attractive for butyrocholinesterase which is believed by some workers[20, 21] to possess no anionic site. The ganglion-blocking activity of nicotinylcholine iodide methiodide could be expected since the distance between the two quaternary nitrogen atoms in this compound is about the same as that which has been found to be optimal for ganglion blocking activity.[2] However, the effect of the nicotinyl derivative is much weaker than that reported for hexamethonium.

The other two bisquaternary compounds although having only one extra atom between the two nitrogens are practically devoid of ganglion blocking action.

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SHORT COMMUNICATIONS

Metabolism of compounds related to ethyl mercaptan

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THE striking *in vivo* antitubercular action of ethyl mercaptan and compounds closely related to it has been established for some years (Davies *et al.*,[1] Brown *et al.*[2]). Recently, Davey and Hogerzeil[3] have reported the success of Etisul (diethyl dithio*iso*phthalate), an ethyl mercaptan derivative, in the treatment of leprosy. Perhaps the most intriguing facets of these observations are (a) the activity is confined to the ethyl mercaptan series, it is not shown by homologous thiols, (b) the antitubercular activity *in vitro* is small and no greater than that of other homologous thiols (Davies *et al.*[1]). This latter fact suggests that the antitubercular action might not be due to ethyl mercaptan *per se* but to some metabolite.

The distribution, excretion and metabolism of ^{35}S-ethyl mercaptan derivatives has been investigated by Snow[4] who showed that following oral dosing of either ethyl thiolbenzoate or diethyl disulphide to guinea-pigs the radioactivity is excreted mainly in the urine and approximately 50 per cent of the administered ^{35}S appeared in the urine as sulphate. Two organic metabolites were also detected, one was ethyl methyl sulphone, the other was not identified. However, neither ethyl methyl sulphone nor a crude mixture containing both metabolites showed any antitubercular activity when tested *in vivo*. The main object of the present work was to study the fate of the carbon moiety of ethyl mercaptan.

^{14}C-Ethyl iodide was converted to the 3:4:5-trichlorobenzoic acid salt of ethyl*iso*thiourea (activity 225 μc/g. salt) from which ^{14}C-ethyl mercaptan was generated by treatment with alkali. The resulting ethyl mercaptan was either oxidized with solid I_2 in alkaline solution to give ^{14}C-diethyl disulphide or benzoylated with benzoyl chloride to give ^{14}C-ethyl thiolbenzoate. Both compounds were purified by distillation. Compounds were dissolved in arachis oil and dosed orally to guinea-pigs through a stomach tube.

For collection of CO_2 and ethyl mercaptan, animals were housed in a metabolism jar of the type described by Berlin *et al.*[5] CO_2-free air was drawn through the apparatus; respiratory ethyl mercaptan was trapped as the $HgCl_2$ complex and CO_2 as K_2CO_3. To minimize losses due to volatilization all tissues were placed in solid CO_2 immediately they were removed from the animal. A small portion of the tissue was then oxidized to CO_2 in a modified van-Slyke type apparatus (Calvin *et al.*[6]) The modification consisted of an extra limb fused on to the oxidation tube at right angles. The CO_2 was collected in N NaOH, precipitated as $BaCO_3$, filtered and counted as such. In most cases, counts were continued for sufficient time to give a reliable error of 2·3 per cent. All counts were corrected to infinite thickness. For absolute determination of ^{14}C a polymer reference source (1 μc/g) was used.

Guinea pigs were killed 48 hr after dosing with ^{14}C-diethyl disulphide and their respiratory gases, urine, faeces and various tissues analysed for ^{14}C. Table 1 shows typical results; it can be seen that approximately 85 per cent of the ^{14}C was recovered, 50 per cent of the administered ^{14}C appeared in the breath as CO_2 and from 3–7 per cent as ethyl mercaptan. The urine contained from 15–25 per cent; the remaining activity was spread throughout the tissues with the liver containing the largest amount.

The nature of the radioactivity in the urine was next studied. No loss of activity occurred when urine was passed through Deacidite FF (OH) but a slight loss was noted after passing through Zeo-Karb 225 (H) indicating the presence of some ^{14}C-cations. After deionisation, samples of urine were chromatographed on paper (ascending) using various solvent systems. The best separations achieved were obtained using n-propanol, n-butanol, water (280 ml, 470 ml, 250 ml). This established that the urine contained two metabolites with R_F 0·40 and 0·75. Snow[4] had previously shown that ethyl methyl sulphone had an R_F 0·6 in aqueous butanol/propanol. It seemed most likely that the spot with R_F 0·75 might also be due to the substance. Since 50 per cent of the activity given to the animal appeared in the breath as CO_2, incorporation of CO_2 into urea was anticipated. Comparison of the chromatograms of urine and urine treated with urease and deionized showed that the latter treatment

removed material with R_F 0·40. Estimation of the ^{14}C in the urea was made, this revealed that 1 per cent of the ^{14}C was incorporated.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY FOLLOWING ORAL DOSING OF ^{14}C-DIETHYL DISULPHIDE TO GUINEA-PIGS

	Experiment 1	*Experiment* 2
Weight of animal	360 g	480 g
Dose of diethyl disulphide	17 mg (7·83 μc)	51 mg (23·5 μc)
Recovery of ^{14}C		
(a) Respiratory CO_2	3·91 μc (50 per cent)	12·81 μc (54·5 per cent)
(b) Breath as EtSH	0·59 μc (7·5 per cent)	0·64 μc (2·7 per cent)
(c) Urine	1·24 μc (15·8 per cent)	5·92 μc (25·2 per cent)
(d) Faeces	0·19 μc (2·5 per cent)	*
(e) Liver	0·16 μc (2·1 per cent)	*
(f) Other tissues (Blood, Spleen, Heart, Lung, Kidney, Stomach, Intestine)	0·53 μc (6·7 per cent)	*
Total Recovery	6·62 μc (84·6 per cent)	19·37 μc (82·4 per cent)

* not measured.

The nature of the spot with R_F 0·75 was next investigated. Urine was collected from guinea pigs dosed orally with ^{14}C-ethyl thiolbenzoate. In all, 243 mg of material (= 145 μc ^{14}C) was administered and 470 ml urine collected. Paper chromatography of this urine again revealed two spots with R_F 0·40 and 0·75. Urea and electrolytes were removed from the urine which was then concentrated by distillation at low temperature (< 30°) and under reduced pressure. The resulting urine concentrate was extracted with $CHCl_3$ and this extract concentrated to 40 ml. This solution was distributed between $CHCl_3$–H_2O in a 50-tube Craig machine. The contents of each tube were concentrated under reduced pressure and low temperature (< 30°) and made up to 2·5 ml for counting. Using an analytical procedure developed by Levi[7] it was shown that the distribution of radioactivity could be represented by the summation of theoretical curves for three substances having K ($CHCl_3$–H_2O) 0·69 (1 part), 2·6 (0·65 part) and 4·9 (0·1 part) (Fig. 1). The difference between the height of the experimental curve in tubes 20 and 21 and the theoretical curve is not fully understood but could be due to the volatility of ethyl methyl sulphone; greater percentage loss occurring in the tubes containing the smaller amounts of this substance. The distribution of ethyl methyl sulphone between $CHCl_3$ and H_2O had previously been shown by Snow[4] to be 0·69. K ($CHCl_3$–H_2O) for ethyl methyl sulphoxide was measured and gave a value of 4·9. Clearly these compounds account for two of the metabolites found in urine. The partition coefficients ($CHCl_3$–H_2O) of β-hydroxyethyl methyl sulphone and β-hydroxy ethyl methyl sulphide were measured: these were found to be 14 (approx.) and 0·6 respectively, thus ruling out the possibility of either of these substances being the third urinary metabolite. The nature of this metabolite is still unknown. Snow[4] found a substance with K($CHCl_3$–H_2O) 2·7 in guinea-pig urine after the animals had been dosed with either ^{35}S ethyl thiolbenzoate or diethyl disulphide. The partition coefficient found in the present work (K = 2·6) is in good agreement with Snow's figure. Moreover, the fact that both substances are neutral, extracted by the same procedure and only appear in the urine after dosing either ethyl thiolbenzoate or diethyl disulphide leaves little doubt that these substances are identical.

These results are in close agreement with those of Snow.[4] There is complete absence of any evidence in the present work suggesting a direct metabolite from the carbon moiety differing from those in which the C–S link is intact. Removal of hydrogen sulphide by desulphydrase action leaving a two-carbon residue seems a possible metabolic path for ethyl mercaptan. Subsequent oxidation of the hydrogen sulphide and metabolism of the two-carbon unit would account for the equivalent production of CO_2 and SO_4^{2-}. The desulphydrases appear to be of general occurrence among higher animals (Fromageot[8]) and the oxidation of hydrogen sulphide to sulphate has been demonstrated to occur

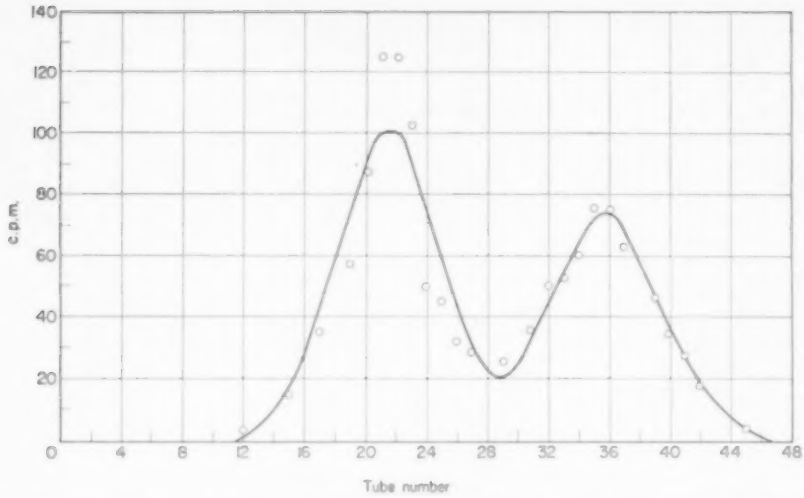

FIG. 1. Counter-current separation of ^{14}C-material. 49 Transfers between $CHCl_3$-H_2O. Points represent determined radioactivity in individual tubes. Curve is theoretical for mixture of three solutes. $K = 0{\cdot}69$ (1 part); $K = 2{\cdot}6$ (0·65 parts); $K = 4{\cdot}9$ (0·1 parts).

in the dog (Dennis and Reed[9]) and rat (Dziewiatkowski[10]). This proposed metabolic route agrees with the findings of Canellakis and Tarver[11] who studied the metabolism of methyl mercaptan in rats. They showed that the sulphur appeared as urinary sulphate while the carbon atom was incorporated into the methyl groups of choline and methionine and into the β-carbon of serine. Failure to find evidence of acidic urinary metabolites suggests that direct oxidation to sulphate via ethane sulphinic and sulphonic acids is not a likely possibility. Snow[4] has previously suggested that the sulphone may arise by methylation of ethyl mercaptan to ethyl methyl sulphide and subsequent oxidation to the sulphone. The finding of small amounts of urinary ethyl methyl sulphoxide agrees with this scheme.

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The metabolism of γ-aminobutyrylcholine

(*Received* 15 *October* 1959)

KURIAKI *et al.*[1] and Kewitz[2] have indicated isolation of γ-aminobutyrylcholine (GABA-Ch) from extracts of the brains of dogs and swine. Pharmacologic evaluation of this choline ester prompted these investigators to suggest that GABA-Ch may be a transmitter of inhibitory pathways in the nervous system. It seemed of interest, therefore, to investigate the possible routes of metabolism of GABA-Ch. The results of these studies with appropriate substrate comparisons are presented in Table 1. The methods employed have been described.[3]

TABLE 1.

Enzyme	Inhibitor		Substrate
1. Cholinesterase		GABA-Ch*	Butyrylcholine
(a) Human plasma	0	40	385
	P†	3	58
(b) Bovine r.b.c.	0	0	5
(c) Mouse brain	0	0	38
(d) Mouse liver	0	26	359
	P†	1	16
(e) Mouse plasma	0	19	348
(f) Cat plasma	0	0	315
2. Amine Oxidases	GABA-Ch*	Putrescine	5-Hydroxytryptamine
(a) Mono-(g pig liver)	0	—	66
(b) Diamine-(hog kidney)	0	22	—

All results are expressed as microlitres of CO_2 (cholinesterase) evolved or O_2 (oxidase) utilized per 30 min per flask, minus enzyme and substrate blanks. Since the primary amine group of GABA-Ch will influence the evolution of CO_2 in the cholinesterase experiments, the reaction was carried out at pH 7·2 employing the chloride-hydrochloride salt. Though the CO_2 evolution–ester hydrolysis relationship is not stoichiometric, the results obtained are indicative of the order of activity of GABA-Ch.

* GABA-Ch (chloride hydrochloride salt) was kindly supplied by Dr. K. Kuriaki.

† P = physostigmine, 10^{-5} M final concentration.

It appears from this preliminary work that few of the enzymes tested were able to catalyse the hydrolysis of the ester. Where hydrolysis was demonstrated, the rate of reaction was small in comparison to the rate of hydrolysis of butyrylcholine. Similar findings have been reported when another naturally-occurring choline ester, imidazole-acrylylcholine,[4, 5] was employed as a cholinesterase substrate.

The catalysed hydrolysis of GABA-Ch by human plasma and mouse liver was inhibited by physostigmine. GABA-Ch did not serve as a substrate for either monoamine or diamine oxidase.

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Possible interpretation and extrapolation of heating experiments with enzymes in the presence of substrates or cofactors

(*Received* 4 *December* 1959)

THE phenomenon of increase of thermal stability of enzymes by substrates has often been observed and used to advantage for enzyme purification. In general, the increase in thermal stability by substrates is believed to be due to protection of an active centre in the enzyme. We have shown recently,[1] however, that under certain conditions substrates or cofactors remarkably decrease the thermal stability of frog liver carbamyl phosphate synthetase. It has occurred to us that this may be a relatively common phenomenon to which little attention has been paid. Increased inactivation of enzymes in the presence of substrate may have occurred often and escaped attention, as it had with us during enzyme purification studies. We have found recently,[2] in testing some twenty enzymes for thermal inactivation, that about half are not affected or are protected by substrates, while the other half are more susceptible to heat in the presence of certain substrates or cofactors. A brief example of the influence of substrate on the thermal stability of glutamine synthetase is given in Table 1. As previously

TABLE 1. THE EFFECT OF SUBSTRATES AND Mg^{2+} ON THERMAL STABILITY OF BRAIN GLUTAMINE SYNTHETASE

Additives (μmoles)		Percentage of initial activity left	
Tris*		Exp. 1	Exp. 2
50	None	52	64
50	5 ATP	20	23
50	20 Mg^{2+}	44	67
50	20 NH_4Cl	47	—
50	20 NH_2OH	—	12
50	20 glutamate	66	80

For experiments 1 and 2, 2·4 and 1·6 units, respectively, of partially-purified beef-brain glutamine synthetase[5, 6] were incubated for 5 min at 60° in 0·5 ml with the indicated components. After cooling, each tube was made up to 1·2 and 4·5 ml for experiments 1 and 2, respectively, and to contain the following, expressed in micromoles (figures in parenthesis are for experiment 2); Tris buffer, pH 7·2, 50 (500); ATP, 15 (50); $MgSO_4$, 20 (200); HN_4Cl or NH_2OH, 50 or (200); glutamate, 100 (500); cysteine (200). The tubes were then incubated for 20 min at 37 °C (30 °C). In experiment 1, NH_4Cl was used, while for experiment 2 the NH_4Cl was replaced by NH_2OH. The extent of the reaction was estimated as described by Elliot[5, 6]. For experiment 2 a succinic anhydride standard was used.

* Tris = Tris(hydroxymethyl)aminomethane.

shown,[1, 2] substrates or combinations thereof may change enzyme stability. It is seen in the table that ATP and hydroxylamine (although the effect by the latter substrate may not be specific) decrease the stability of the enzyme, while glutamate protects. In the experiments shown in the table, ATP when combined with Mg^{2+}, or with Mg^{2+} and glutamate, or with Mg^{2+} and NH_4Cl, provided protection. Recently Inagaki[3] has shown that glutamic dehydrogenase is less stable to heat in the presence of reduced diphosphopyridine nucleotide than in its absence, and Jenkins *et al.*[4] have shown a similar effect with glutamic-aspartic transaminase and aspartate. However, these workers have not commented on the possible broad biological significance of this phenomenon or on its possible use as a tool for enzymic studies. Substrate interaction may protect an active centre or, changing the geometry of the enzyme, may make other groups more susceptible to inactivation by physical agents. Since the decrease in enzyme stability to heat with substrates is a relatively common phenomenon and since

decreased enzymic activity induced by substrates has been noted by us with X-rays, ultraviolet light and ultrasonic waves (to be presented elsewhere), it seems possible that this may reflect a general effect which can provide a working hypothesis for certain aspects of poorly-understood problems such as tachyphylaxis, drug habituation, chemotherapy, fever and allergic phenomena. The administration of a drug, or of a substrate in a large excess, may result in increased destruction of an enzyme particularly at high temperatures and if other necessary components are not present at concentrations which will protect the enzyme. Under these conditions, either after resynthesis of the enzyme(s) or after removal of end products, the drug will be effective again (tachyphylaxis). Either enzyme binding, or destruction, or both, or slow resynthesis of the enzyme, may require higher doses to produce similar effects due to affinity considerations (drug habituation). It is well known that many drugs, for example, streptomycin, penicillin, certain sulphonamides and isoinazid can be more effective at lower temperatures. Fever might influence biological systems in an unsuspected manner. Examples in immunology possibly related to the phenomena under considerations, are the cold agglutinins and the higher precipitate obtained at 0 °C than at 37 °C in cross reactions.

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Enzymic oxidation of psilocine and other hydroxyindoles

(*Received* 21 *December* 1959)

IT has recently been shown that the gill plates of *Mytilus edulis* L. contain an oxidase that acts on 5-hydroxyindoles, e.g. 5-hydroxytryptamine, bufotenine and 5-hydroxytryptophan; in the oxidation of these substances yellowish-brown pigments are formed.[1]

We have recently been able to compare the enzymic oxidation of bufotenine with that of its 4-hydroxy and 6-hydroxy analogues. The 4-hydroxy analogue of bufotenine is psilocine, a psychotropic amine naturally occurring in the fungus *Psilocybe mexicana* Heim,[2] where it is found together with its phosphate ester, psilocybine.

It was found that both hydroxyindoles were oxidized by the *Mytilus* preparation. The rate of oxidation of the 6-hydroxy derivative was similar to that of bufotenine; a faint yellowish-orange colour appeared during the reaction. With psilocine as substrate, oxygen uptake was more rapid than with bufotenine or with 5-hydroxytryptamine, and a blue colour appeared in the reaction. The rapid phase of the oxidation was over when about one atom of oxygen per molecule of added psilocine had been consumed. The contents of the manometer flask then showed a deep blue colour, with an absorption maximum at 625 mμ.

We also had an opportunity of studying the *N*-1-methyl derivatives of both bufotenine and of psilocine. The 5-hydroxy compound was oxidized at about the same rate as bufotenine. The *N*-1-methyl derivative of psilocine was oxidized more slowly than psilocine itself, and the development of the blue colour was less rapid. These observations show that the substrates of the *Mytilus* oxidase

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are not restricted to 5-hydroxy indoles. We therefore propose the name "hydroxyindole oxidase" for the enzyme.

The rapid oxidation of psilocine, together with the development of the deep blue colour, is of particular interest. It is suggested that in the enzymic reaction of the 4-hydroxyindole an *o*-quinonoid compound is formed. The oxidation of the 5-hydroxy and the 6-hydroxy indoles may lead to the formation of *p*-quinones.

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Cerebrospinal fluid and bradykinin release

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A RECENT investigation[1] showed a correlation between bradykinin release and the activation of plasmin in rat and bovine plasma. Various human body fluids contain an activator of plasminogen or its precursor.[2] Normal human cerebrospinal fluid was found to contain only the proactivator of the fibrinolytic system.[2] More recently Chapman and Wolff[3] showed that cerebrospinal fluid from patients with an active central nervous system (CNS) disease contracted smooth muscle or developed such activity when incubated with a bovine globulin fraction. Bioassay showed similar properties to vasodilator peptides[4] derived from plasma proteins, such as bradykinin[5] which is released by protease action. It became of interest to decide whether cerebrospinal fluid from patients with CNS disease contains sufficient amounts of protease to release bradykinin or whether the reaction involves activation of a fibrinolytic type.[6]

Cerebrospinal fluid from five patients* was investigated for protease activity using the synthetic substrates† benzoyl-l-arginine methylester (BAMe) and toluenesulfonylarginine methylester (TAMe) and the fibrin plate method.[6, 9] At most, only traces of protease activity could be detected with the synthetic subtrates. In one case, however, (multiple sclerosis) a protease/esterase activity was observed with 0·01 M-TAMe (no activity with BAMe) and 0·08 ml per ml of the cerebrospinal fluid in 0·02 M-phosphate pH 7·7. trypsin† (5 μg per ml), approximately ten times higher activity was obtained. When trypsin was added after incubation of the substrate with the cerebrospinal fluid no activity developed, showing that the substrate was used up and that although low this sample had a detectable

* The author wishes to thank Dr. E. Hillbom and Dr. Y. O. Alanen, the Psychiatric and Neurological Clinic of the University of Helsinki for their interest and co-operation.

† The substrates were kindly supplied by the Research Division of the Cleveland Clinic Foundation and the trypsin by Dr. T. Astrup, the Biological Institute of the Carlsberg Foundation, Copenhagen.

protease action. No inhibitory activity towards these substrates was found to be present in the cerebrospinal fluids when added to samples incubated with trypsin in concentration up to 0·2 ml per ml.

When applied to the fibrin plates a weak protease activity appeared in some of these cases, showing that, if present, the amount of free protease acting on fibrin was very low (the fibrin plate was sensitive to 0·01 μg trypsin in 0·03 ml applied).[8] However, when streptokinase was added to the cerebrospinal fluids a strong activation appeared on the standard plates.[8] On the heated fibrin plates[9] activity appeared only after addition of a bovine plasminogen preparation.[1] While the standard fibrinogen preparation contains plasminogen,[8] the latter is destroyed by heating the clotted fibrin plates.[9] The results therefore indicate that while free protease is absent or low in the cases of cerebrospinal fluid tried, a precursor of a plasminogen activator is present, in agreement with the findings of Albrechtsen *et al.*[2]

It could now be demonstrated (Fig. 1) that the presence of all three components, cerebrospinal fluid, streptokinase and plasminogen, was necessary to develop smooth muscle contracting activity from a bovine plasma substrate.[1] As previously shown, this substrate contains plasminogen that can

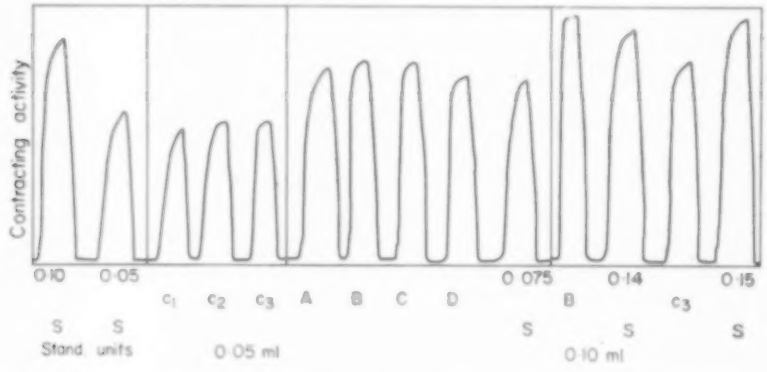

FIG. 1. Cerebrospinal fluid from patients with schizophrenia (A, B) and multiple sclerosis (C, D) was incubated with a bovine plasma substrate[1] in the presence of streptokinase (4 hr at 38°, pH 7·8). After an alcohol extraction smooth muscle (guinea pig ileum) contracting activity was assayed in comparision to standard bradykinin units[10]. In control experiments with substrate and cerebrospinal fluid (schizophrenic) (c_1), substrate alone (c_2), and substrate and streptokinase (c_3), 42·5 per cent less smooth muscle contracting activity was released. (Plasma concentration 0·25 ml per ml.)

be activated to form free plasmin. With an incomplete fibrinolytic system (Fig. 1, controls) significantly less bradykinin was formed. This is apparently due to the slowed spontaneous activation of plasminogen in the bovine substrate.[1]

A freshly prepared globulin fraction of bovine plasma precipitated at 50 per cent ammonium sulphate concentration and dialysed[8] was also used to study the effect of the cerebrospinal fluid upon bradykinin release. On incubation with an equal volume of the globulins (0·5 ml) for 13 minutes at 38° pH 7·7, no smooth muscle contracting activity developed. In a control experiment normal bradykinin activity developed (approximately 8·5 units per ml plasma) when released with 1 mg heated *Bothrops jararaca* venom (containing a protease/esterase).[10, 11] After about 10 min of incubation all activity disappeared owing to the presence of a bradykinin destroying enzyme in a fresh globulin preparation. Since longer incubation time could not be applied, the study of low protease action upon the bradykinin precursor in this type of substrate would be unsuitable. The acid heated plasma substrate applied above contains less inhibitors and no bradykinin destroying activity. From this substrate bradykinin can be released with relatively small amounts of trypsin or snake venom or by the activation of plasminogen.[1]

Cerebrospinal fluid had no effect directly upon the isolated guinea pig ileum (sensitive to 0·05 units of bradykinin) when applied in amounts up to 0·5 ml in a 3–4 ml bath of Tyrode solution. Nor did

any effect appear upon the addition of streptokinase (200 mg) and globulin preparation (0·5 ml). When, after 10 min incubation of the globulins with streptokinase and cerebrospinal fluid from a schizophrenic, snake venom was added, smooth muscle contracting activity appeared normally after 1 min of incubation[10] then slowly disappeared.

These experiments do not exclude the possibility that free protease may appear under certain conditions in the cerebrospinal fluid of patients with CNS disease. In the cases tried, however, the protease activity if present would not be sufficient to release bradykinin under the above conditions. A detailed account of this work will be published elsewhere.

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Papers to be published in future issues

V. C. DEWEY, M. R. HEINRICH, D. G. MARKEES and G. W. KIDDER: Multiple inhibition by 6-methylpurine.

P. ROSENBERG: *In vivo* reaction by PAM of brain cholinesterase inhibited by Paraoxon.

L. MIKKONEN, T. TUOMINEN and E. KULONEN: Collagen fractions in lathyritic rats.

T. FREDRIKSSON and G. TIBBLING: Inhibition of cholinesterase with methylfluorophosphoryl choline and -carbocholine; spontaneous return of activity.

W. P. BURKHARD, K. F. GEY and A. PLETSCHER: Inhibition of diamine oxidase *in vivo* by hydrazine derivatives.

J. P. GREEN and M. DAY: Heparin, 5-hydroxytryptamine and histamine in neoplastic mast cells.

A. HORITA and W. R. MCGRATH: The interactions between reversible and irreversible monoamine oxidase inhibitors.

C. H. GALLAGHER: The effect of pyrrolizidine alkaloids on liver enzyme systems.

J. H. KOCH and C. H. GALLAGHER: The effects of neuro-muscular blocking agents on mitochondria.

A. L. TAPPEL: Inhibition of electron transport by antimycin A, alkyl hydroxynaphthoquinones and metal coordination compounds.

R. G. WIEGAND and J. D. TAYLOR: Kinetics of plasma drug levels after sustained release dosage.

P. EMMELOT and C. J. BOS: On the Mg^{++} dependency of the mitochondrial ATPase activation by two azocarcinogens and a related cytotoxic strylquinoline.

C. C. KRATZING, J. R. DUNSTONE, N. P. MADSEN, PATRICIA MACDONALD and HELEN BELL: Variations in rat liver calcium and magnesium.

BO HOLMSTEDT and F. SJÖQUIST: Pharmacological properties of γ-aminobutyrylcholine, a supposed inhibitory neurotransmitter.

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MULTIPLE INHIBITION BY 6-METHYLPURINE*

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Abstract—Inhibition of the growth of *Tetrahymena pyriformis* by 1-deaza-6-methylpurine is not readily reversed by adenine. This purine analog also causes the death and eventual lysis of the cells. While it does not have a lethal effect, 6-methylpurine (6MeP) is a more potent inhibitor of growth, and is reversed by high concentrations of adenine. Low concentrations are ineffective. Synergistic reversing effects on inhibition by 6MeP are exhibited by combinations of small amounts of adenine (10–15 μg/ml) with acetate. Metabolites such as sterols and phospholipids produced biosynthetically from acetate are capable of replacing the mixture of acetate plus adenine (10–15 μg/ml). Peptides, but not amino acids, give additional release of inhibition in the presence of lipid materials. It is suggested that 6MeP interferes specifically with the production of acyl or amino acyl compounds, probably by affecting the synthesis or utilization of ATP, as well as by interfering with adenine utilization for nucleic acid synthesis.

THE inhibition of the growth of *Tetrahymena pyriformis* by a number of analogs of adenine has already been described.[1, 2] Although adenine is not a nutritional requirement of the ciliate, since it is obtainable from guanine,[3] nevertheless specific inhibition of adenine utilization is obtained with either 2-azaadenine § or 8-azaadenine or 1-deazaadenine. Guanine has no reversing effect on these inhibitors.

The additional effect of causing death of the ciliates in cultures partially inhibited by 1-deazaadenine,[2] as distinguished from simple inhibition of growth, stimulated interest in other deazapurines. In the course of this work the compound 7-methyl-1,3,4-imidazopyridine, which could be regarded as 1-deaza-6-methylpurine, was independently synthesized by a route different from that previously reported[4] and tested. While this compound was somewhat less inhibitory than 1-deazaadenine, it possessed the property of causing death of the organisms. Unlike 1-deazaadinine, it was not readily reversed by adenine, of which it may also be regarded as an analog.

For comparative purposes 6-methylpurine (6MeP)[5] was obtained and tested.[6] This compound has proved to be the most inhibitory for *Tetrahymena* of the purine analogs so far tested (6-mercaptopurine is without activity in this system). 6-Propylpurine‖ had less than 1/100 of the activity of 6MeP. Since 6MeP did not cause the death and eventual lysis of the cells (which complicates turbidimetric measurements of growth) characteristic of the deaza-compounds, it was studied in more detail than

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§ Unpublished results.

‖ Obtained through the courtesy of G. B. Elion.

1-deaza-6-methylpurine. Adenine proved to be relatively ineffective in reversal, although its effect could be greatly potentiated by the addition of acetate or certain products formed by biosynthetic pathways from acetate, as well as by addition of peptides. No inhibition was produced by 2-hydroxy- or 2-mercapto-6-methylpurine.

MATERIALS AND METHODS

Tetrahymena pyriformis W was cultivated in medium A[7] containing Tween 80 (10 mg/ml). Acetate and adenylic acid were omitted from this medium in certain experiments. Growth was measured turbidimetrically[8] after incubation for 4 days in a slanted position at 25°.

Sterols were dissolved in the Tween 80 used in preparing the basal medium. The various phospholipids were homogenized, with the aid of a small amount of Tween 80, using a Potter–Elvehjem apparatus.

Lipositol was prepared from commercial soy bean lecithin by the method of Woolley[9] and phosphatidyl ethanolamine by the method of Scholfield and Dutton.[10] Inositol phosphatides were also prepared using techniques described by Folch.[11] Crude glycolipids were extracted from beef spinal cord lipids using hot ethanol. The enzymatically hydrolyzed vitamin-free casein (VFC-EH) was obtained from Nutritional Biochemicals Corp.

By catalytic reduction of 2-amino-3-nitro-4-picoline[12] and subsequent ring closure of the resulting diamine with 98 per cent formic acid, 7-methyl-1,3,4-imidazopyridine was obtained in 65 per cent yield. Melting point 151·5–152° (corrected); literature,[4] 146–147°.

Analysis calculated for $C_7H_7N_3$: C, 63·14; H, 5·30; N, 31·6. Found: C, 63·04; H, 5·39; N, 31·6.

RESULTS

Reversal by adenine of the inhibition produced by 6MeP was not strictly competitive: inhibition indices varying from 0·023 to 1·46 were obtained, depending upon the adenine concentration in the medium. However, a sufficiently high concentration of adenine reversed the inhibition (Fig. 1).

The above results suggested that some compound other than adenine might be more effective in reversal. Adenosine proved to be less active than adenine, but the activity of adenosine-3′-phosphate (or adenosine-5′-phosphate) was approximately equal to that of adenine. None of the amino acids, vitamins or nucleic acid derivatives in the medium, when tested in increased concentrations, had any effect. In addition, natural materials were also investigated. "Distillers' solubles" was found to have the greatest reversing effect. Since the purine content of this material is low, non-purine compounds were implicated as having a reversing effect on the inhibition.

The fact that "distillers' solubles" was used as a starting material for the isolation of mevalonic acid,[13] suggested the possibility that the latter might be active. However, the addition of mevalonic acid* to media containing 6MeP enhanced the inhibitory effect of the purine analog to a slight extent. In turn, these results suggested that the acetate in the medium might have an effect on the inhibition, and as may be seen from Table 1, acetate was synergistic with adenine. Acetate alone, even at high concentrations, was only slightly effective, but when combined with amounts of adenine

* Obtained through the courtesy of Karl Folkers.

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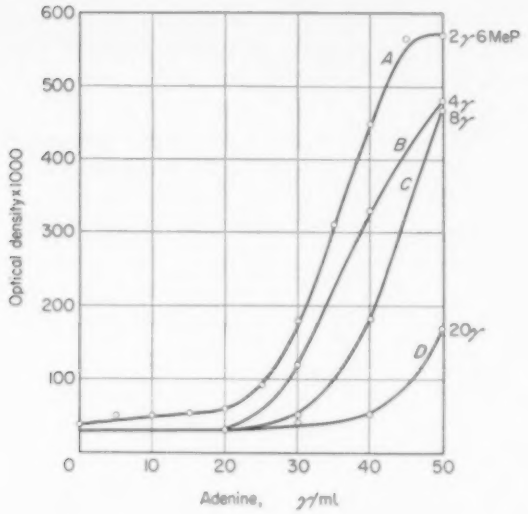

FIG. 1. Response of *Tetrahymena pyriformis* to adenine in presence of 6-methylpurine. Medium A minus acetate and adenylic acid. Curve A, 6-MeP 2 μg/ml; curve B, 6-MeP 4 μg/ml; curve C, 6-MeP 8 μg/ml; curve D, 6-MeP 20 μg/ml.

(e.g., 10–15 μg/ml) which were ineffective in themselves, a decided reversal was obtained.

The effect of acetate was limited in that relatively large amounts were required and beyond a certain point (1 mg/ml) further additions of acetate produced little further reversal of inhibition (Table 1, col. 1). The fact that "distillers' solubles" produced reversal without addition of adenine suggested that compounds other than acetate might be concerned. For these reasons substances known to be produced biosynthetically from acetate were tested.

The most active of these substances proved to be sterols (Table 2). Only those sterols (cholesterol, stigmasterol, β-sitosterol, ergosterol) having the intact side chain were active. Alterations in the side chain, as seen in the genins, or degradation to the

TABLE 1. SYNERGISTIC EFFECT OF ACETATE AND ADENINE ON INHIBITION BY 6-METHYLPURINE

Acetate (μg/ml)	Adenine (μg/ml)								
	0	5	10	15	20	25	30	40	50
	Optical Density								
0	0·04	0·04	0·04	0·06	0·10	0·16	0·27	0·43	0·48
100	0·05	0·05	0·09	0·12	0·14	0·17	0·26	0·48	0·55
250	0·07	0·09	0·13	0·17	0·20	0·24	0·30	0·46	0·52
500	0·09	0·12	0·17	0·22	0·24	0·26	0·31	0·46	0·52
1000	0·10	0·16	0·20	0·25	0·26	0·28	0·32	0·47	0·56
2000	0·13	0·17	0·20	0·24	0·26	0·29	0·34	0·53	0·61

Medium A minus acetate. adenylic acid: 6MeP 2 μg/ml.

level of the bile acids, adrenal hormones or sex hormones produced inactive or actually inhibitory compounds.[15] Acetylation of the 3-position in cholesterol reduced activity slightly, while opening the B ring (as in calciferol) completely inactivated the compound. Squalene*, a proposed precursor of sterols, had little if any activity.

Since stigmasterol has been used to supply the sterol requirement of another ciliate, *Paramecium aurelia*,[16] it was used in most of the present experiments. More detailed

TABLE 2. EFFECT OF STEROLS ON 6-METHYLPURINE INHIBITION

Steroid	μg/ml					
	0	5	10	25	50	100
			Optical Density			
Sitosterol	0·19	0·26	0·29	0·36	0·44	0·45
Ergosterol		0·24	0·29	0·32	0·37	
Stigmasterol		0·23	0·25	0·30	0·36	
Cholesterol		0·22	0·26	0·30	0·40	0·42
Calciferol		0·20	0·19	0·19	0·20	
Digitonin		0·17	0·17	0·14	0·17	
Smilagenin		0·16	0·17	0·17	0·18	
Squalene	0·14		0·13	0·13	0·11	0·08
Hydroxybisnorcholenic acid	0·10	0·11	0·07	0·10	0·13	
Cholesteryl acetate	0·08	0·08	0·12	0·13	0·30	0·32
Dehydroepiandrosterone	0·10			0·06	0·04	0·03
Cortisone	0·14		0·08	0·06	0·04	0·03

6-MeP 2 μg/ml, acetate present

TABLE 3. COMPARISON OF EFFECTS OF β-SITOSTEROL AND OF STIGMASTEROL IN REVERSAL OF INHIBITION BY 6-METHYLPURINE

	β-Sitosterol (μg/ml)							
	0	5	10	15	20	30	40	50
				Optical Density				
	0·04	0·13	0·19	0·20	0·22	0·24	0·25	0·25
+ Acetate	0·08	0·24	0·33	0·36	0·36	0·41	0·43	0 44
				Stigmasterol				
	0·04	0·10	0·11	0·12	0·12	0·12	0·13	0·14
+ Acetate	0·07	0·21	0·23	0·26	0·28	0·31	0·32	0·33

Medium A minus acetate, adenylic acid; 6MeP 2 μg/ml.

comparisons in which various sterols were tested at low concentrations in the same experiment showed that β-sitosterol was the most active sterol (Table 3). Stigmasterol, cholesterol and ergosterol were less active, but about equal to one another.

Like acetate, the sterols also were of limited effectiveness. While smaller amounts were required and activity was present even without the addition of adenine, there was a point beyond which added sterol was without effect. Since the presence of acetate improved growth over that obtained with sterols, it was apparent that still other substances are active. It was considered possible that the unknown materials might be

* Obtained through the courtesy of Karl Folkers.

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products peculiar to the metabolism of the ciliate. Therefore, a lipid extract of *Tetrahymena* was chromatographed on silicic acid by the method of Fillerup and Mead.[17] The most active fraction obtained was the one containing the phospholipids.

Various commercial preparations of animal and plant phospholipids (lecithins) were then tested for activity. Considerable variation was found between preparations of supposedly similar material. For this reason, one of the more active preparations was chromatographed on silicic acid by the method of Marinetti *et al.*,[18] but none of the phospholipid fractions showed any significant activity alone, or when all were combined; on the other hand, when stigmasterol was present in the medium, all the

TABLE 4. EFFECT OF LIPIDS ON INHIBITION BY 6-METHYLPURINE

Additions	μg/ml							
	0		10		50		100	
	a	b	a	b	a	b	a	b
	Optical Density							
Lipositol	0·03	0·11	0·3	0·21	0·04	0·26	0·07	0·25
Phosphatidyl Ethanolamine	0·03	0·10	0·02	0·20	0·03	0·27	0·03	0·15
Inositol Phosphatides	0·03	0·11	0·03	0·18	0·04	0·23	0·03	0·21
Glycolipids	0·04	0·11	0·04	0·17	0·05	0·24	0·05	0·28
Fatty Acids								
soy bean	0·03	0·13	0·03	0·16	0·05	0·24	0·07	0·25
C_{20}unsat.	0·03	0·12	0·03	0·19	0·04	0·22	0·03	0·23

6-methylpurine 2 μg/ml. a = medium A minus acetate and adenylic acid. b = same as a plus stigmasterol, 50 μg/ml.

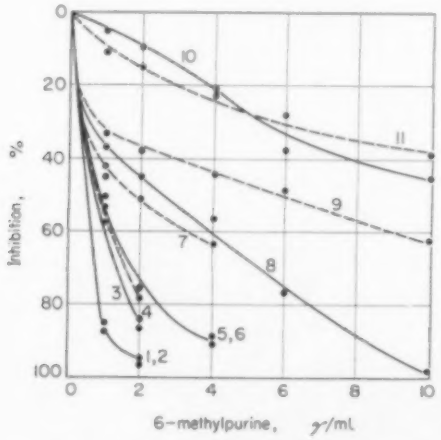

FIG. 2. Effect of combinations of active compounds in reversal of inhibition by 6-methylpurine; Curve 1, no addition; Curve 2, adenine (10 μg/ml); Curve 3, acetate (1 mg/ml); Curve 4, enzymatically hydrolyzed vitamin-free casein (1 mg/ml); Curve 5, lecithin (100 μg/ml); Curve 6, stigmasterol (50 μg/ml); Curve 7, casein hydrolyzate (Curve 4) + stigmasterol (Curve 6); Curve 8, lecithin (5) + stigmasterol (6); Curve 9, lecithin + stigmasterol + casein hydrolyzate; Curve 10, lecithin + stigmasterol + casein hydrolyzate + adenine; Curve 11, lecithin + stigmasterol + casein hydrolyzate + adenine + acetate.

fractions were active. It would appear that the more active of the commercial samples were those which contained a certain proportion of sterols.

In view of the fact that all fractions showed some activity, in the presence of sterols, no attempt was made to characterize the material present in each. Instead, standard isolation procedures were employed to prepare compounds such as lipositol,[9] phosphatidyl ethanolamine[10] Folch inositides[11] or glycolipids. Table 4 shows the ineffectiveness of these compounds in the absence of sterol and their effectiveness in its presence. There was little difference between the various lipids and, in fact, a mixture of unsaturated fatty acids (C_{20} unsaturated) gave essentially the same effect as the conjugated lipids. Inositol, on the other hand, had no effect on the inhibition, either alone or in combination with other compounds. Single long chain unsaturated fatty acids showed little activity, but combinations, either from natural sources or prepared from the isolated fatty acids, were effective.

These results suggest that 6MeP interferes with the activation of acetate in the synthesis of compounds of higher molecular weight. Since amino acids are activated by

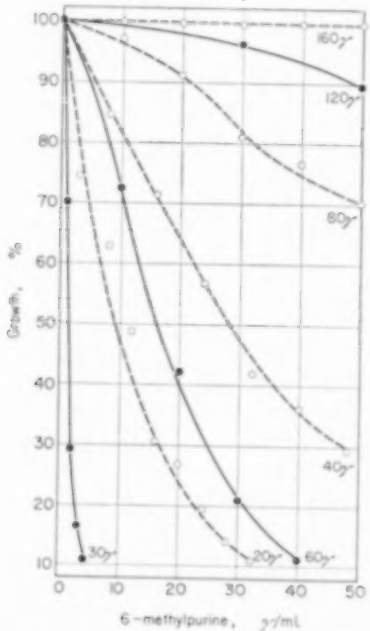

FIG. 3. Response of *Tetrahymena pyriformis* to 6-methylpurine. Open circles, medium A minus acetate and adenylic acid. Solid circles, medium A minus acetate and adenylic acid, plus stigmasterol 50 μg/ml, lecithin 100 μg/ml, and enzymatic hydrolysate of vitamin-free casein 1 mg/ml. Numbers beside curves indicate adenine concentration/ml.

similar mechanisms,[19] it seemed possible that peptides might also have an effect on 6MeP-inhibition. Therefore, an enzymatic hydrolysate of vitamin-free casein was tested. From Fig. 2 it is apparent that peptides were also capable of antagonizing the effect of 6MeP, particularly in the presence of sterol and lecithin. While crude lecithin was used in these experiments, entirely similar results were obtained with the more

purified preparations. Single simple dipeptides, such as leucylglycine, caused only small effects.

Included in the figure are the results obtained when adenine (10 μg/ml) was added in the presence of these various compounds. Under these conditions, in contrast to the addition of adenine alone, the effect was marked, especially at lower concentrations of 6MeP. The addition of acetate instead of adenine had no effect, but when added in the presence of adenine at 10 μg/ml a slight stimulation occurred. Therefore, it must be assumed that still other products of acetate metabolism (such as carotenoids and coenzyme Q) are capable of influencing the response to 6MeP.

As shown in Fig. 3, adenine in sufficiently high concentration completely reversed the inhibitory effect of 6MeP. Concentrations of adenine above 160 μg/ml were not tested, since adenine itself becomes inhibitory above this level. Also, it is apparent from the figure that the presence of sterols, lecithin and peptides considerably enhanced the activity of adenine.

A few experiments similar to those described above have been carried out using 6-methyl-deazapurine as the inhibitor. The pattern of reversal appears to be very similar to that of 6MeP, in that the effectiveness of adenine was enhanced by the presence of acetate, sterols and lipids. In the absence of adenine these compounds were relatively ineffective, as was adenine alone. Death of a certain proportion of the cells occurred even in the presence of the reversing compounds, so that definite quantitative results cannot be presented. Death and lysis of the cells, instead of clarifying the cultures, led to increased turbidity through the release into the medium of fat globules, glycogen granules or other formed elements.

DISCUSSION

A possible interpretation of the above experiments is that 6MeP exerts a multiple inhibitory effect, having as its source the inhibition of the activation of acetate and amino acids, and perhaps other similar compounds as well. These effects are mediated through adenosine triphosphate (ATP), so that 6MeP may prove to be a specific analog of adenine in the synthesis or utilization of ATP or other coenzymes, as well as in nucleic acid synthesis. The inactivity of 6-methylguanine or 6-methylpyrimidine points to a very definite specificity of inhibition.

While 6-mercaptopurine did not inhibit *Tetrahymena*, it is a potent inhibitor in other systems. Thus, in *Escherichia coli* cultures, Bolton and Mandel[20] reported inhibition of acetate incorporation by 6-mercaptopurine, and in cultures of cells of Sarcoma 180, Biesele[21] found that mitotic aberations produced by 6-mercaptopurine or 6-thioguanine[22] were relieved by the presence in the medium of either coenzyme A or thioctic acid (α-lipoic acid) or both. Biesele[21] has suggested that there may be interference with the metabolism of 2-carbon compounds in such inhibited cultures.

That acetate and therefore sterols may have an even more intimate connection with ATP-metabolism is demonstrated by the fact that stigmasterol overcame the inhibitory effect of dinitrophenol[23]. Here the action of the sterol appears to have been on the uncoupling of phosphorylation by dinitrophenol, rather than on ATP-synthesis, although the two inhibitors might prevent acetate activation through different mechanisms.

The results obtained with 6MeP resemble those described by Elion[24] using synergistic combinations of inhibitory analogs. In both cases, much larger concentrations

of the reversing compounds were required to release the inhibition than is the case with inhibitors assumed to have a single effect. This finding serves as another illustration of the possible multiple nature of the inhibition produced by 6MeP.

Although 6MeP is toxic in higher animals[25] and relatively ineffective against Sarcoma 180[26, 27] it may prove useful as a tool in studies of adenine metabolism in certain systems.

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COLLAGEN FRACTIONS IN LATHYRITIC RATS

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Abstract—The effect of β-aminopropionitrile ("Lathyrism") on collagen was studied. Albino rats were fed with a diet containing 73 per cent sweet peas, and the bone changes resulting were ascertained by x-ray studies. The collagen fractions were prepared from skin and bone by extraction with disodium phosphate solution, acid citrate solution, and by gelatinization.

The alkali-soluble collagen was 5 times greater in cases of lathyrism. The solubility of non-collagenous proteins was also increased. The total collagen content was not altered.

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USING the electron microscope, Follis and Tousimis[1] have demonstrated that fibrous collagen is deposited more slowly in the cartilage of lathyritic rats than in control rats. The hydroxyproline content is unaltered, but a disturbance in the synthesis of collagen is indicated by other observations: there is a delay in the formation of mature fibres in subcutaneous abscesses,[2] as well as in the formation of hydroxyproline both in croton oil pouches[2] and in growing animals;[3] and there is a decrease in the tensile strength of collagen in healing wounds.[4, 5] On the other hand, it has been claimed that the collagen content in skin is normal.[6] These statements lead us to study collagen fractions of biological interest.

MATERIALS AND METHODS

The selected rats (Wistar) weighed from 30 g to 44 g initially. They were separated into two groups of ten animals each, the first being fed on the sweet pea diet, the second on the control diet.[4] These diets contained approximately 73 per cent of *Lathyrus odoratus* seeds and of *Pisum sativum* peas, respectively. The animals were part of a larger experiment on diets, and five rats from each group received iproniazid, which aggravates lathyrism,[7] while the remainder received methionine sulphoxide, which does not. The diet lasted 22 days, at the end of which time the rats weighed from 38 to 69 g in the lathyritic group, and from 49 to 79 g in the control group. The development of lathyrism was then established by individual x-ray examinations.

The hair and subcutaneous fat having been removed, the skins were cut up finely with scissors, extracted with ether, and air-dried. For the extraction, four samples were formed in each group by combining two or three skins (giving samples weighing 1·06–2·62 g and 2·34–5·42 g, in lathyritic and control groups respectively). Some thigh and shin bones were also extracted by a similar technique.

The extraction fluids were those of Harkness *et al.*,[8] and a Microid flask-shaker (Griffin and Tatlock Ltd.) was used. The whole extraction was carried out at 2–4° for 8–22 hr, using 10 parts of saturated disodium phosphate solution to one part of

the sample (v/w): it was repeated five times, and the supernatant extracts were separated at each stage by centrifugation for 30 min at 2500 rev/min. The extracts were combined, filtered through Jena Glass Filter G3, and saturated disodium phosphate solution was added to make a total volume of 250 ml: the resulting solution was dialysed for four days against running tap water (10–14°), in cellophane bags. The precipitate was separated by centrifugation for one hr at 2500 rev/min, and was suspended in 25 ml of 0·08 N NaOH.

The residue was extracted again by a similar technique, using 0·25 M citrate buffer, pH 3·8, and the collagen soluble in acid citrate was precipitated by dialysis, and collected.

The insoluble collagen was then obtained by autoclaving the residue twice with 100 ml distilled water for 1½ hr at 1 atm, the fluids being filtered while still hot. Autoclaving a third time did not add appreciably to the extracted nitrogenous material. A residue of about 20–25 per cent of the original weight remained.

The total soluble protein and the citrate-soluble collagen were calculated from nitrogen content (estimated by Kjeldahl combustion and distillation). The analysis of the other collagen fractions was based on the determination of hydroxyproline.[9] Both carbazole[10] and orcinol[11] reactions were used for the determination of the uronic acid.

RESULTS AND DISCUSSION

The results are collected in Table 1. There is no difference between the analyses of uronic acid from different bone samples: the significance of this is still uncertain. But

TABLE 1. SKIN FRACTIONS IN EXPERIMENTAL LATHYRISM OF RAT (PER CENT OF AIR-DRIED SAMPLE)

Group	N	Soluble in saturated disodium phosphate solution					Acid-soluble collagen	Insoluble collagen
		Protein	Uronic acid		Hydroxyproline (×7·15)			
			Carb.	Orc.	Total	Precip.		
Lathyrus	4	27·7	0·20	0·16	3·15	1·07	0·48	19·4
Control	4	18·9	0·14	0·14	0·62	0·30	0·18	19·0
t		2·75	2·56	—	6·87	5·1	1·8	—
P		<0·05	<0·05	—	<0·001	<0·01	—	—

the difference in alkali-soluble collagen from healthy or lathyritic bone can be confirmed ($P<0·05$). When the hydroxyproline was determined in the disodium phosphate extract by accident without prior hydrolysis, the values so obtained were also greater for lathyritic materials ($P<0·001$ for skin; $P<0·01$ for bones). The proportion of this "directly acting" hydroxyproline of the total hydroxyproline was clearly less in cases of lathyrism.

On dialysis, a smaller proportion of the hydroxyproline soluble in disodium phosphate was precipitated in the lathyritic than in the control samples; and when this precipitate was suspended in pH 3·8 citrate buffer, after storage at 5°, it was found

that only the lathyritic samples were still soluble, which suggests differences in precipitation properties.

The recent reports on the solubilization of collagen in rats treated with acetonitrile[12], as well as on the increased solubility in M NaCl of collagen from chick embryos treated with aminopropionitrile[13,14], are in good agreement with the observations cited in this paper.

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INHIBITION OF CHOLINESTERASE WITH METHYLFLUOROPHOSPHORYLCHOLINE AND CARBOCHOLINE: SPONTANEOUS RETURN OF ACTIVITY

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Abstract—The spontaneous reversal of cholinesterase activity has been studied following inhibition with methylfluorophosphorylcholine, methylfluorophosphorylcarbocholine (3 : 3-dimethylbutoxyphosphoryl fluoride) and *iso*propoxymethylphosphoryl fluoride (Sarin). Aceto- and butyrocholinesterase have been used as enzyme sources, and the reversal of enzyme activity has been studied at 4°, 25° and 35°. Inhibition produced by the two phosphorylcholines is readily reversible in contrast to that produced by Sarin. This is explained in terms of formation of an enzyme–inhibitor complex of another type than the one produced by Sarin. The results also supports the theory that butyrocholinesterase lacks an anionic site, and they confirm the previous observation that in intact animals methylfluorophosphorylcholines produce symptoms of shorter duration than ordinary organophosphorus cholinesterase inhibitors.

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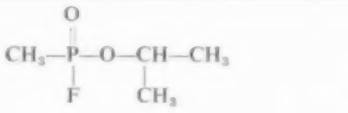
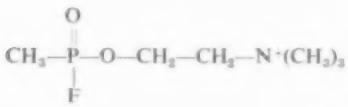
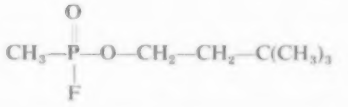
PREVIOUS studies[3, 4] of methylfluorophosphorylcholines (Table 1) gave the impression that animals intoxicated with these compounds showed symptoms which were of shorter duration than those produced by ordinary organophosphorus cholinesterase inhibitors, such as Sarin. Furthermore, cumulative effects could not be demonstrated in repeated toxicity tests on mice. It was therefore assumed that the enzyme inhibition was more reversible in the case of methylfluorophosphorylcholines, probably due to a more rapid spontaneous hydrolysis of the enzyme–inhibitor complex.

However, Enander[2] could not, in experiments *in vitro*, reactivate human erythrocyte cholinesterase inhibited by methyl fluorophosphorylcholines with the aid of a series of reactivators. On the other hand, it was possible to reverse neuromuscular block in the rat nerve–diaphragm preparation with different reactivators.[5] However, enzyme studies on homogenized rat diaphragm muscle could not reveal a reactivation of acetocholinesterase inhibited by methylfluorophosphorylcholine, while Sarin inhibited enzyme could be reactivated.[11] Thus, the reversal of the neuromuscular block seems to be produced by mechanism other than reactivation of the enzyme activity, possibly due to interaction with receptors.

Methylfluorophosphorylcholines are considered to produce a phosphorylation of the esteratic site of acetocholinesterase with liberation of fluoride ions.[10] But owing to their positively-charged nitrogen group they probably also react with the anionic site of the enzyme with the formation of an ionic bond.[10] This may explain why they are very potent acetocholinesterase inhibitors, and this interaction with the anionic site may give the enzyme–inhibitor complex particular properties in regard to stability.

In view of this it seemed to be of interest to investigate the spontaneous hydrolysis of the enzyme–inhibitor complex with methylfluorophosphorylcholine as inhibitor and make comparisons with Sarin, using both aceto- and butyrocholinesterase as enzyme sources. It was also decided to use methylfluorophosphorylcarbocholine as

TABLE 1.

Substance	Formula	pI_{50}*	
		Erythrocytes	Plasma
Sarin		8·8	8·4
Methylfluorophosphorylcholine		10·0	8·4
Methylfluorophosphorylcarbocholine		9·0	8·3

* From Tammelin[9].

inhibitor, an analogue to the former compound but containing a carbon atom instead of the quaternary nitrogen. This compound, though having a closely similar spacial arrangement, can be assumed to be considerably less attracted to the anionic site (Table 1).

METHODS

Enzyme sources. Human erythrocytes were used as source of acetocholinesterase and separated from fresh, heparinized blood by centrifugation. They were washed three times in 0·9% saline and then haemolysed in distilled water, the original volume being diluted ten times. Finally the sample was diluted with an equal volume of Michel's buffer (0·02 M sodium barbital, 0·004 M KH_2PO_4, 0·6 M KCl and 0·1 N HCl to pH 8·14).

As source of butyrocholinesterase a preparation of Cohn's fraction 4 : VI was chosen (manufactured by Kabi AB, Stockholm, Sweden). Twenty mg were diluted in equal parts of distilled water and Michel's buffer to a final volume of 100 ml.

Inhibitors. The inhibitors used, synthetized in this institution, appear in Table 1 together with their pI_{50}-values (the negative logarithm of the molar concentration causing 50 per cent inhibition of enzyme activity). They were diluted in the enzyme solutions to a final concentration of $5 \cdot 10^{-7}$ M, a concentration found to cause complete inhibition of the enzyme activity. The disappearance of the excess of inhibitor, i.e. its hydrolysis, was controlled in the following way. Samples were withdrawn at 24 hr intervals and mixed with equal volumes of the enzyme stock solutions. The enzymic

activity of this mixture was then measured. The excess of inhibitor was regarded as lost when there was no further decrease in enzyme activity.

Enzyme activity determinations. The enzyme activities were estimated with the electrometric method described by Tammelin.[9] Acetylcholine iodide (final concentration $0{\cdot}73 \times 10^{-2}$ M) was used as substrate, and all measurements were performed at 25 ° following incubation during 30 min. All enzyme activity values were calculated as percentages of the activity of the enzyme stock solutions. Corrections for the spontaneous hydrolysis of the substrate were not found to be necessary.

General procedure. The enzyme stock solutions and the test solutions were stored in darkness at the following temperatures: 4 °, 25 ° and 35 °. At 24 hr intervals samples were withdrawn and the activities determined (two independent measurements were always performed). The pH of the solutions was also determined daily with the aid of a type pHM 23 c pH-meter (manufactured by Radiometer, Copenhagen). Two independent series of experiments were performed.

RESULTS

Spontaneous loss of enzyme activity following storage

There was a slight loss in enzyme activity of the stock solutions following storage, particularly at the two higher temperatures, and the acetocholinesterase preparation was the least stable (Table 2). For this reason daily determinations of the enzyme

TABLE 2. SPONTANEOUS LOSS OF ENZYME ACTIVITY FOLLOWING STORAGE AT DIFFERENT TEMPERATURES, EXPRESSED IN PER CENT PER 24 HR

Temperature	(°C)	4	25	35
Acetocholinestrase	(%)	1–2	6–7	8–9
Butyrocholinesterase	(%)	<1	3–4	4–5

activity of the stock solutions, stored at the same temperatures as the test solutions, were performed. These values were regarded as 100 per cent values for the relevant temperature and time and used as the basis for the other calculations.

Variations in the pH of stock solutions following storage

The stock solution of the acetocholinesterase preparation had initially a pH of 8·03–8·05, and the pH of the butyrocholinesterase preparation was 8·10–8·12. Following storage at 4 ° there was a daily decrease in pH of about 0·03–0·04 units in case of the acetocholinesterase preparation, while the decrease in pH did not amount to more than 0·04–0·06 units during 10 days in the case of the butyrocholinesterase preparation. At 25 ° the daily decrease was, respectively, 0·1 and 0·03 units, and about the same decreases were also found following storage at 35 °.

Disappearance of the excess of inhibitor (*Table* 3)

The excess of methylfluorophosphorylcholine was found to have disappeared within 24 hr following storage at all the different temperatures. In case of methylfluorophosphorylcarbocholine the excess was found to be lost within 48 hr at 4 ° and within 24 hr at 25 ° and 35 °. The excess of Sarin disappeared within 5 days

following storage at 4 ° and within 24 hr following storage at the other two temperatures.

Spontaneous return of enzyme activity following inhibition

These results are summarized in Figs. 1–3. Following storage at 4 ° for 8 days there is full spontaneous return of enzyme activity in only two cases; i.e. with butyrocholinesterase and methylfluorophosphorylcholine and -carbocholine as inhibitors

TABLE 3. CALCULATED HALF-LIFE PERIODS ($t_{\frac{1}{2}}$) AND TIME UNTIL DISAPPEARANCE OF EXCESS OF INHIBITOR AT DIFFERENT TEMPERATURES

	4 °		25 °		35 °	
	$t_{\frac{1}{2}}$	Disappearance of excess	$t_{\frac{1}{2}}$	Disappearance of excess	$t_{\frac{1}{2}}$	Disappearance of excess
Methylfluorophosphorylcholine	2–2·5 hr	<24 hr	6–8 min	<24 hr	2–2·5 min	<24 hr
Methylfluorophosphorylcarbocholine	55–70 hr	<48 hr	2–2·5 hr	<24 hr	30–40 min	<24 hr
Sarin	70–90 hr	<5 days	4–5 hr	<24 hr	1–1·5 hr	<24 hr

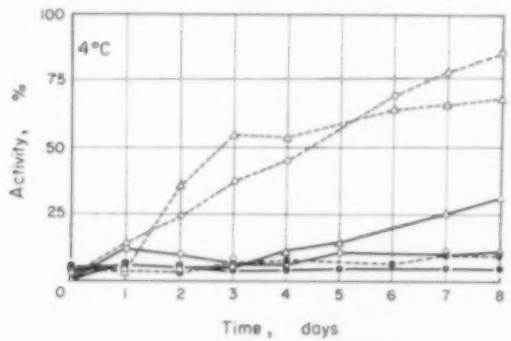


FIG. 1. Spontaneous return of enzyme activity following storage at 4 °: ——— acetocholinesterase, - - - - - butyrocholinesterase. ● Sarin (*iso*propoxymethylphosphoryl fluoride). ○ methylfluorophosphorylcholine. ▲ methylfluorophosphorylcarbocholine.

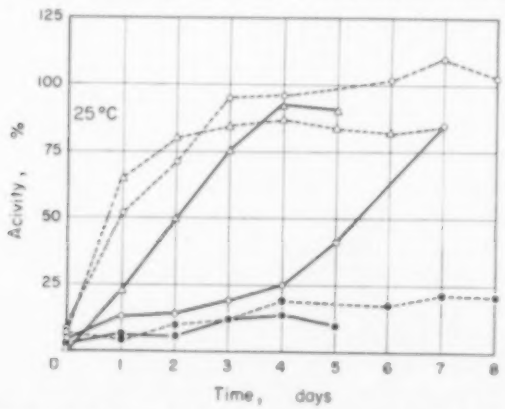


FIG. 2. Spontaneous return of enzyme activity following storage at 25 °. Symbols as in Fig. 1.

(Fig. 1). Acetocholinesterase inhibited with the carbocholine analogue shows a partial return of activity. Following storage at 25 ° there is return of activity except when Sarin is used as inhibitor (Fig. 2), and the reversal of inhibition is rapid in the other four cases with one exception, i.e. acetocholinesterase inhibited with methylfluorophosphorylcholine. The same tendency is found following storage at 35 °, but here is also some return of the enzyme activity in the case of butyrocholinesterase inhibited with Sarin (Fig. 3).

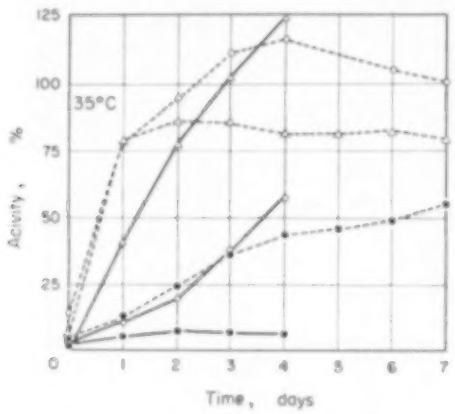


FIG. 3. Spontaneous return of enzyme activity following storage at 35 °. Symbols as in Fig. 1.

DISCUSSION

Following storage there is a spontaneous loss of enzyme activity and a decrease in pH of the stock solutions; the exact nature of these two processes will not be discussed in this paper. These changes are, however, of importance for the present measurements. The decrease in pH means that the sensitivity of the method used to record enzyme activity also decreases, but only in the case of acetocholinesterase.[6] Errors resulting from this have been reduced by performing daily measurements of the activity of the stock solutions and using these activity values as a basis for the calculations. These corrections account also for spontaneous loss of enzyme activity.

Larsson[7] has determined the second-order rate constants for the alkaline hydrolysis (pH 8·5) of the compounds at 25 ° and 35 °. From these values the half-life periods have been calculated, corrected for actual pH, temperature and ionic strength (Table 3). In those cases, where our method of estimating the rate of disappearance of the excess of inhibitor is sensitive enough, the loss is somewhat faster than the corresponding calculated values indicate. It can be assumed that this is due to the presence of enzymes catalysing the hydrolysis, i.e. the cholinesterase itself and/or phosphorylphosphatases.

Osterbaan *et al.*[8] have shown that when DFP reacts with butyrocholinesterase the fluorine is first split off and then one of the *iso*propoxy groups, presumably resulting in two linkages between the phosphorus atom and the enzyme. It is reasonable to assume that an analogous reaction also may occur in the case of Sarin, i.e. the fluorine and the alkoxy group are split off. The methyl group is less reactive than the alkoxy group, and it is therefore unreasonable to suppose that it will be displaced.

If the two phosphorylcholines react in the same way, i.e. by splitting off the flourine and the choline or carbocholine group, this would result in a phosphorylated enzyme, being the same for both compounds and identical with the one produced by Sarin. This assumption means that the spontaneous return of enzyme activity would occur at the same rate in all the three cases. As this is not the fact, the phosphorylcholines must produce a phosphorylated enzyme of different structure. This interpretation is also supported by the fact that acetocholinesterase inhibited with phosphorylcholines could not be reactivated with PAM, while Sarin inhibited enzyme could be reactivated.[2] It thus seems as if the phosphorylcholines only react with the enzyme by splitting off the flourine, leaving the alkoxy group intact, which was originally suggested by Tammelin.[10] This simple linkage to the enzyme may explain the comparatively rapid spontaneous return of enzyme activity.

The rate of return of enzyme activity is the same for both phosphorylcholines, when butyrocholinesterase is used as enzyme source. This indicates that the compounds react with this enzyme in an analogous way. When acetocholinesterase, however, is used as enzyme source, the return of enzyme activity occurs at different rates, suggesting a different type of reaction with the enzyme. This may be explained by the choline analogue forming an ionic bond with the anionic site of the enzyme, owing to its quaternized structure. The results thus also support the theory that butyrocholinesterase lacks an anionic site.[1] This conclusion can also be drawn from the pI_{50}-values[9] (Table 1). The slight difference in rate of return of activity between carbocholine inhibited butyro- and acetocholinesterase may depend on its polarized structure, resulting in a weak attraction to the anionic site of the latter enzyme.

Finally, the present results are consistent with the initial observation[3, 4] that phosphorylcholines produce symptoms of considerably shorter duration than other organophosphorus cholinesterase inhibitors.

Acknowledgement—We wish to express our thanks to Prof. G. Ljunggren for his kind interest in this investigation and to Miss Britt Nilsson for skilful technical assistance.

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ADDENDUM:

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HEPARIN, 5-HYDROXYTRYPTAMINE, AND HISTAMINE IN NEOPLASTIC MAST CELLS*

J. P. GREEN† and MARGARET DAY‡

(*Received* 23 *November* 1959)

Abstract—During culture *in vitro* of an ascitic mast-cell tumor of the mouse, P-815, three strains have been developed, two of which were obtained by a cloning-technique. One of these pure strains is near diploid, the other tetraploid. All strains maintain their intracellular levels of heparin, histamine, and 5-hydroxytryptamine (5-HT) in culture. The levels of these three substances and the incorporation of S^{35}-sulfate, glucosamine-1-C^{14}, and glucose-U-C^{14} into heparin have been compared in the cells both in the mouse and in culture. Strains vary in their contents of these compounds and in their capacity to incorporate S^{35}-sulfate and glucosamine-1-C^{14} into heparin, but in all cases the amounts of heparin, 5-HT, and histamine are higher in culture than when the same strain of cells are grown in the mouse; similarly, the incorporation of S^{35}-sulfate into heparin is higher in culture. The amounts of the amines, especially of 5-HT, are proportional to the amount of heparin. The three substances are found in the same intracellular fraction. By paper chromatography mast-cell heparin has been resolved into three fractions. The concentration of one of these is proportional both to the amount of heparin, as determined by bioassay, and to the levels of amines, especially 5-HT. This component of heparin is not present in bovine heparin. It was also noted that sulfate and glucosamine in heparin turn over at the same rate. The half-life of heparin in the mast-cells is approximately two and one half days.

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INTRODUCTION

A TRANSPLANTABLE mouse mastocytoma, P-815,[2] containing 5-hydroxytryptamine (5-HT), histamine,[3,4] and heparin[5,6,7] maintains its levels of these compounds after continuous growth in culture.[4,8] The development of a cell line, termed the T-line, having higher levels of 5-HT and histamine than the original line (0-line) in culture, has already been described.[4] From the T-cells, two clonal sub-lines X-1 and X-2, were obtained[4] by two consecutive cloning procedures.[9] In this paper are recorded some cytological characteristics of these cells, their capacity to take up S^{35}-sulfate, glucose-U-C^{14}, and glucosamine-1-C^{14}, and to incorporate them into heparin, and the turnover-rate of sulfate and glucosamine in heparin. In addition, some aspects of the relationships among 5-HT, histamine, and heparin have been explored.

METHODS

ALL the mast cell lines were maintained in (AKR × DBA/2) F1 hybrid mice or in DBA/2 mice, the tumor being routinely carried in the ascitic form. The culture techniques and the medium in which the cells were grown have already been described in

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† Senior Research Fellow of the United States Public Health Service.

‡ Recipient of a postdoctoral fellowship provided by a grant from the Squibb Institute for Medical Research.

detail;[4] the medium differed only in the addition of Neomycin (0·1 mg/ml) and Mycostatin (Squibb, 50 units/ml). An inoculum of 1–3 $\times$ 10^4 cells/ml could be used provided that 30 ml of 2 per cent tris(hydroxymethyl)-aminomethane in 0·15 N-HCl were added to every liter of medium to give a final of pH of 7·4. This inoculum was used routinely in the later experiments. Under these conditions, a mean generation-time of 10 to 12 hr was obtained. In experiments in which the cells were incubated with radioactive isotopes, a cell-inoculum of 3 $\times$ 10^5 cells/ml was used. Magnesium chloride was substituted for magnesium sulfate in the medium when the uptake of S^{35}-sulfate was studied, and when glucose-U-C^{14} was added to the medium, the concentration of the glucose of the medium was reduced to one-half that normally used (i.e., to 0·5 g/l.). The levels and sources of radioactive isotopes used in culture were 0·5 mc (0·5 mmoles) of S^{35}-sulfate (obtained from the Oak Ridge National Laboratory); 0·001 mc (0·001 mmole) of glucosamine-1-C^{14}, kindly donated by Charles Pfizer and Co. through the courtesy of Dr Domenic G. Iezzoni; 0·005 mc (0·02 mmole) of glucose-U-C^{14}, and 0·005 mc (0·005 mmole) of glucuronate-6-C^{14} (the last two substances were obtained from Nuclear Chicago). For studies of the uptake of S^{35}-sulfate by mast cells in the mouse, 1·0 mc of S^{35}-sulfate was injected intraperitoneally.

Cells were removed from the mouse by aspiration of the peritoneal fluid and, like those obtained from culture, collected by centrifugation, and washed three times with ice-cold 0·9 per cent sodium chloride.

To count chromosomes, cells were removed from the mice four days after inoculation and five hr after injecting 1·25 μg of colchicine per g body weight. Cells in culture were incubated for 15 hr with 3 μg colchicine/ml of medium.

5-HT-levels were determined on the isolated heart of *Venus mercenaria* and histamine was assayed on the isolated ileum of the guinea pig, as described previously.[4]

To extract heparin, the mast cells were sonicated, placed in a boiling water-bath to coagulate protein and, after cooling, placed in cellophane membranes which had been heated for three days at 80°.[5] Pancreatin or crystalline trypsin (10 mg/5 $\times$ 10^8 cells) was added to each bag, and the proteolysis-dialysis carried out against 0·02 M-Tris buffer, pH 8·6, for 24 hr. A further 24-hr dialysis was routinely carried out against running tap-water; in experiments in which the dialysates were collected, dialysis was carried out against distilled water, and the dialysates were concentrated *in vacuo*. The non-dialyzable material was heated, centrifuged, and five volumes of acetone were added to the supernatant fluid. After refrigeration over-night, the precipitate was dissolved in a small volume of water. The solution was shaken with three volumes of trichlorotrifluoroethane[10] ($CF(Cl_2)$–$CCl(F_2)$; "Genetron 113", Allied Chemical and Dye Corp.) to yield, after centrifugation, an upper aqueous phase containing all the heparin, a gel-like intermediate layer of protein, and the heavy solvent layer devoid of heparin. The aqueous phase was collected and residual Genetron 113 removed by gentle heating.

In the assays, heparin was compared with a standard preparation (1 unit = 10 μg; Nutritional Biochemicals Corp.) either by metachromasia or by anticlotting activity, or both; hereafter, the anticlotting activity is referred to as "bioassay". Metachromasia was determined with Azure A by the method of Jaques *et al*,[11] (absorption at 490 mμ in a Beckman DU-spectrophotometer was employed). For bioassay of heparin, blood was taken from a dog by venepuncture, mixed with nine volumes of 4 per cent

sodium citrate, and the plasma was collected. The clotting system consisted of 0·2 ml of plasma; 0·2 ml (12 mg) of fibrinogen (Armour) in 0·005 M-Tris Buffer, pH 7·3; 0·2 ml (0·2 mg) of thrombin (Upjohn); and 0·1 ml of the unknown solution or of a standard solution of heparin. With some samples of plasma, it was necessary to reduce the concentration of thrombin by one-half to obtain an anticoagulant effect with the standard heparin. Thrombin was added last, at which point timing was begun. The tubes were tilted gently every 15 sec until a clot was formed. For each assay, at least two concentrations of heparin were employed. The unknown was diluted and its heparin content estimated by comparison with the standard solution, which usually contained 0·03 units of heparin. This method proved more sensitive than the method described in the U.S. Pharmacopeia, but the values obtained by the two methods were in good agreement.

Heparin was chromatographed on Whatman No. 3 paper in *iso*propyl alcohol-0·04 M-Ammonium Formate (35 : 65). Metachromasia on paper was detected by dipping the dried paper into 0·1 per cent Azure A in ethanol—water—1 N-acetic acid (5 : 4 : 1) and then into the same solvent mixture without Azure A. Papers were also dipped into an alkaline solution of silver nitrate[12] after preliminary experiments had shown that several hours after being dipped and dried, heparin formed a violet color on paper. The radioactivity of all samples was measured in a liquid scintillation counter, and radioactivity on paper was detected with a Geiger–Mueller tube. Hydrolysis of heparin was carried out in a sealed tube for 48 hr in 3 N-HCl, and the hydrolysate, together with a glucose-marker, was chromatographed in 1-propanol—ethyl acetate—water (70 : 10 : 20).[12] After the paper dried, it was dipped in an alkaline solution of silver nitrate. Cells were fractionated in 0·25 M-sucrose by the method of Schneider and Hogeboom.[13]

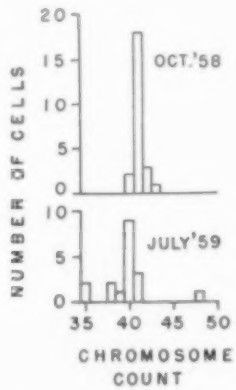

FIG. 1. Chromosome counts on X-2 cells maintained in the mouse.

RESULTS

Cytology. Chromosome counts on X-1 and X-2 cells were made in October, 1958, four months after the development of the two clones, by Dr. T. S. Hauschka of the Roswell Park Memorial Institute. In July, 1959, comparable counts were made by Dr. Julian J. Jaffe of this department. The X-2 line in the mouse retained its near diploid chromosome number after repeated transfers during this nine-month period, as shown in Fig. 1. On the other hand, the polyploid X-1 cells (Fig. 2), after successive

transfers in mice during the same period, showed marked instability, as revealed by a widened distribution of chromosome counts in the population. However, the X-1 cells which had been kept in culture during the nine-month period remained stable (Fig. 2).

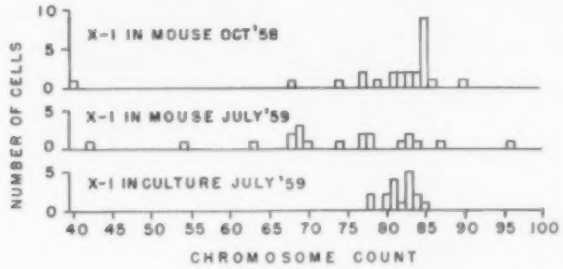

FIG. 2. Chromosome counts on X-1 cells maintained in the mouse and in culture.

Cell size. As shown in Figs. 3a and 3b, the polyploid X-1 cells are larger than the near-diploid X-2. They are also less uniform in size.

Neoplastic virulence. After twenty generations in culture, the cloned mast cell lines, X-1 and X-2, retained their neoplastic virulence when injected into mice; this is shown in Fig. 4. During culture, the large polyploid (X-1) cells, exhibited slightly greater infectivity than did the original parent strain of cells (O-cells)[4], whereas X-2 cells showed the same infectivity as the O-cells. The "T-line", from which both X-1 and X-2 were developed, had the same infectivity as the original tumor. Necropsies on all animals showed no grossly different anatomical effects among the cell lines.

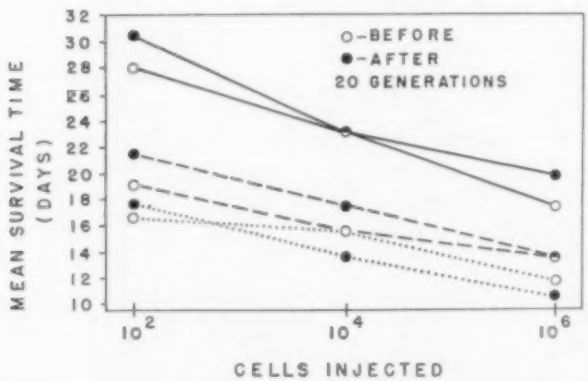

FIG. 4. The virulence of different cell-lines before and after culture. X-1 cells ———; original cells (O-cells) – – –; X-2 cells · · · · ·.

Incorporation of glucose-U-C^{14} into heparin by X-1 *and* X-2 *cells in culture.* Both clones incorporated glucose-U-C^{14} into heparin at the same rate (Fig. 5). That the non-dialyzable material was indeed heparin was shown in several ways: (a) it had anticoagulant activity; (b) it was metachromatic, both in solution and on paper after chromatography; (c) all the radioactivity of the non-dialyzable material

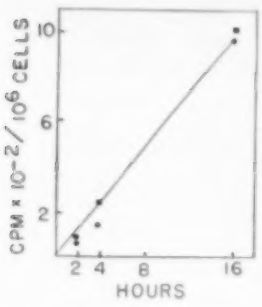

FIG. 5. Incorporation of glucose-U-C^{14} into heparin by X-1 (■——■) and X-2 (●——●) cells, in culture.

was precipitated by the addition in aqueous solution of each of several quaternary salts (cetyltrimethylammonium bromide, cetylpyridinium chloride, and cetyldimethylbenzylammonium chloride) known to precipitate acidic and sulfated polysaccharides, and also by 4-amino-4′-chloro-diphenyl, a precipitant of only highly sulfated polysaccharides.[14]

The acid-hydrolysate of the heparin showed a radioactive spot at R_f 0·83 coincident with a brown spot which formed after dipping in an alkaline solution of silver nitrate. Authentic glucosamine, chromatographed separately and in admixture with the hydrolysate, gave the same R_f value. In this solvent system, galactosamine had an R_f of 0·45.

Failure of X-1 *and* X-2 *cells to take up glucuronate*-6-C^{14}. During an incubation period of 24 hr, neither strain of cells took up any glucuronate-6-C^{14}.

Incorporation of glucosamine-1-C^{14} *into heparin by* X-1 *and* X-2 *cells in culture.* Glucosamine-1-C^{14} was incorporated into heparin by both strains of cells, X-1 showing the more rapid uptake (Fig. 6). Paper chromatography of the acid-hydrolysates of the heparin (as above) showed a single radioactive spot corresponding to glucosamine.

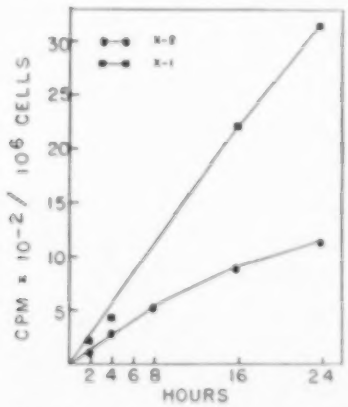

FIG. 6. Incorporation of glucosamine-1-C^{14} into heparin by X-1 cells (■——■) and X-2 (●——●) cells, in culture.

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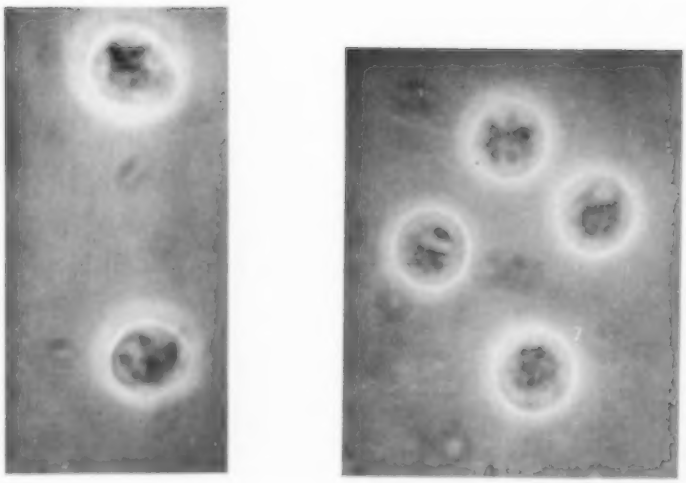

(a) (b)

FIG. 3. (a) The polyploid X-1 cells and (b) the near-diploid X-2 cells, as seen with the phase microscope.

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Uptake of S^{35}*-sulfate by mast cells* in vitro *and in culture and its incorporation into heparin.* The uptake of S^{35}-sulfate and its incorporation into heparin by X-1 and X-2 cells, both in the mouse and in culture, was measured. Fig. 7 indicates that the isotope was rapidly taken up by the X-1 cells *in vivo*. As early as two hr after its injection, the major portion of the S^{35}-sulfate was in the form of heparin; by 16 hr, 95 per cent of the isotope was accountable as heparin. The amount of dialyzable S^{35}-sulfate in the cell reached a peak at four hr and then fell. The dialyzable material had different R_f values from those of the non-dialyzable material, and metachromasia was not exhibited either in solution or on paper after chromatography.

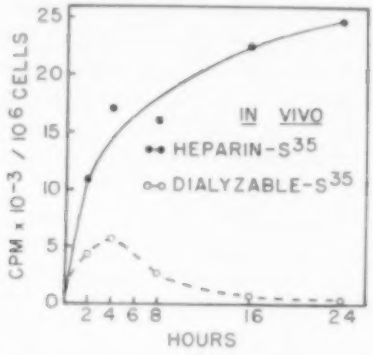

FIG. 7. The uptake of S^{35}-sulfate by X-1 mast cells in the mouse.

Fig. 8 shows the uptake of S^{35}-sulfate by X-2 cells in culture and the incorporation of the isotope into heparin. Unlike the same strain of cells in the mouse, the cells in culture continued to take up the radioactive isotope, so that at 24 hr more than 50 per cent of the radioactivity was still dialyzable. The simplest interpretation of the difference between the experiments *in vivo* (Fig. 7) and *in vitro* (Fig. 8) is that the cells incubated in culture are exposed to a persistently high level of S^{35}-sulfate in the medium, whereas *in vivo* the inorganic S^{35}-sulfate is being rapidly excreted.[15]

Failure of X-2 *cells in culture to secrete heparin.* After the growth of the X-2 cells in culture, the nutrient medium containing the S^{35}-sulfate was collected and concentrated *in vacuo*. All radioactive material was dialyzable, non-metachromatic, and when chromatographed on paper had the R_f value of inorganic sulfate. Radioactive heparin could not be detected.

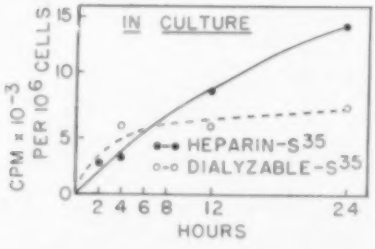

FIG. 8. The uptake of S^{35}-sulfate by X-2 mast cells, in culture.

Absence of heparin in the horse-serum used in the culture medium. Horse serum (100 ml) was digested with trypsin (100 mg) and extracted for heparin after dialysis and concentration *in vacuo* of the non-dialyzable material. None was detectable either by metachromasia in solution or after paper chromatography. The amount of heparin in this sample of horse serum was less than 0·01 units/ml. The concentration of heparin in the culture medium was thus less than 0·002/units per 10^6 cells; this was too low to account for the levels of heparin in the cell, even if the cells could concentrate preformed heparin.

Failure of X-1 *cells to take up heparin-*S^{35} *or heparin-*C^{14} *in culture.* To determine whether the cells could take up heparin from the medium, radioactive heparin was prepared from X-1 cells that had been incubated with either S^{35}-sulfate or glucosamine-1-C^{14}. The labeled heparin was added to two separate cultures of X-1 cells. After 4, 6, and 24 hr, cells were harvested, washed in the usual way, and their radioactivity was measured. In no instance was there any detectable radioactivity in the cells. Under these conditions, therefore, the mast cells were unable to take up heparin.

Half-lives of heparin-$S^{35}O_4$ *and of heparin-glucosamine-*C^{14}. Twenty-four hr after the addition of S^{35}-sulfate or of glucosamine-1-C^{14} to the X-1 cells in culture, and every 24 hr thereafter for one week, the cells were harvested and a cell-count was taken. The cell-population was then divided into two portions. One aliquot was extracted for heparin and the radioactivity was measured; the other was placed in an isotope-free medium. The loss of radioactivity with time, corrected for cell-multiplication, is shown in Fig. 9. The turnover-rate of glucosamine and of sulfate in heparin were the same. The half-life of heparin was two and one-half days.

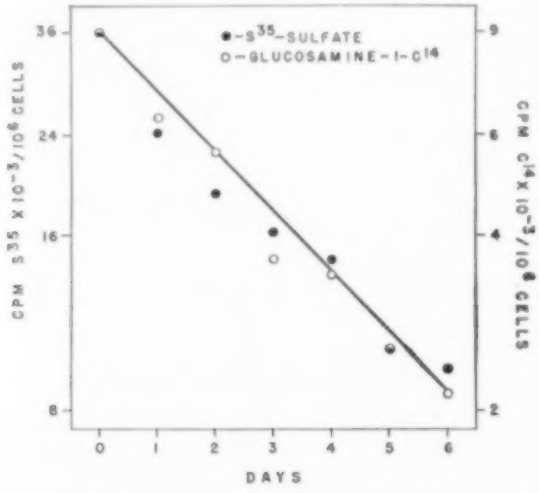

FIG. 9. Turnover of S^{35}-sulfate (○——○) and glucosamine-1-C^{14} (●——●) in heparin by X-1 cells.

Levels of 5-HT *and histamine.* Over a six-month period, both 5-HT and histamine were simultaneously determined in three cell lines (T, X-1, and X-2) grown in the mouse as well as in tissue culture (Table 1). Consistently, in each cell-line, cells grown

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in culture had greater amounts of the amines than the same cells grown in the mouse. The levels of the two amines varied widely (as is evident from the standard deviation), even in the genetically stable X-2 cells. These cells, in comparative experiments, usually, though not always, showed lower levels of amines than the larger X-1 cells, as suggested by the data in Table 1.

TABLE 1. CONTENT OF 5-HT AND HISTAMINE OF THREE STRAINS OF MAST CELL TUMORS IN THE MOUSE AND IN CULTURE

Cell Line	In Mouse		In Culture	
	5-HT	Histamine	5-HT	Histamine
T	0·17 ± 0·05(7)	0·25 ± 0·08(6)	0·26 ± 0·10(15)	0·40 ± 0·36(12)
X-1	0·32 ± 0·14(12)	0·47 ± 0·26(13)	0·89 ± 0·37(10)	0·66 ± 0·31(11)
X-2	0·16 ± 0·15(4)	0·28 ± 0·14(3)	0·64 ± 0·25(6)	0·42 ± 0·20(5)

The numbers within parentheses denote the number of samples.

Relative levels of amines and heparin. As the levels of amines varied in any one cell-line widely between experiments (Table 1) a comparison of these levels among groups of cells was carried out at the same time. In Table 2 are recorded the values of 5-HT, histamine, and heparin, the last measured by two methods which are seen to agree well. In every instance, cells grown in culture had greater amounts of all three compounds than cells grown in the mouse, an increase especially remarkable with the X-1 cells. These polyploid X-1 cells, it should be added, usually have greater amounts of the three substances than the smaller, diploid X-2 cells, but in this experiment the X-1 cells in the mouse had significantly lower levels than the X-2 cells. In general, levels of 5-HT and histamine paralleled each other; and the levels of the amines, especially 5-HT, varied as the levels of heparin varied.

TABLE 2. LEVELS OF 5-HT, HISTAMINE AND HEPARIN IN FOUR DIFFERENT STRAINS OF MAST CELLS, IN THE MOUSE AND IN CULTURE

Cell Strain	In Mouse				In Culture			
	5-HT μg	Histamine μg	Heparin Bioassay units	Heparin Azure A units	5-HT μg	Histamine μg	Heparin Bioassay units	Heparin Azure A units
O	0·01	0·19	0·01	0·02	—	—	—	—
T	0·10	0·12	0·03	0·04	0·20	0·40	0·11	0·13
X-1	0·05	0·08	0·03	0·02	1·05	0·31	0·13	0·15
X-2	0·39	0·35	0·09	0·09	0·70	0·50	0·18	—

All values are for 10^6 cells.

The lack of a more precise relationship between and among these compounds can be explained, at least in part, by the variability of the levels of these amines in a single cell-line at any one time, especially if grown in the mouse. In Table 3 are shown the levels of the compounds in X-1 cells which had been inoculated into mice at the same

TABLE 3. LEVELS OF 5-HT, HISTAMINE AND HEPARIN IN X-1 CELLS COLLECTED FROM MICE ON THE SAME DAY

Sample	5-HT μg	Histamine μg	Heparin-$S^{35}O_4$		
			units*	cpm	cpm/μg
1	0·32	0·66	0·091	12,400	134,000
2	0·48	0·46	0·101	15,800	156,000
3	0·56	0·71	0·106	15,000	142,000
4	0·80	1·15	0·121	17,400	143,000
5	0·90	0·92	0·124	17,300	139,000

All values are for 10^6 cells.
* Determined by metachromasia with Azure A.

time and harvested one week later. Sixteen hr before collecting the cells, each animal was given S^{35}-sulfate intraperitoneally. The amounts of histamine detected were more variable than those of 5-HT, although the latter also showed a range greater than one would expect from experimental error. Least variable were the levels of heparin. Here again the amount of heparin appeared more closely correlated with the amount of 5-HT than with that of histamine. Also, the amount of heparin in X-1 cells was nicely reflected in their capacity to incorporate S^{35}-sulfate into heparin.

The parallel in X-1 cells between their heparin-content and the labeling by S^{35}-sulfate of the heparin suggested that a similar relationship may exist within the other cell lines. Accordingly, the amounts of heparin (and of 5-HT and histamine), and the labeling of heparin by S^{35}-sulfate, were measured in three other strains in the mouse (Table 4). Again the amount of heparin was correlated with the amount of amines,

TABLE 4. LEVELS OF 5-HT, HISTAMINE AND HEPARIN AND ITS CHROMATOGRAPHIC DISTRIBUTION IN DIFFERENT STRAINS OF MAST CELLS

Cell Strain	5-HT μg	Histamine μg	Heparin		Chromatographed		Heparin-$S^{35}O_4$
			units*	cpm†	R_f 0·00‡	R_f 0·76‡	R_f 0·94‡
O	0·03	0·11	0·04	3900	70	140	25
T	0·12	0·35	0·14	4200	250	50	3
X-2	0·18	0·38	0·20	14,200	300	300	15

The values given are for 10^6 cells.
* Determined by bioassay.
† Counted in liquid scintillation counter.
‡ Paper chromatograms counted directly with probe.

especially 5-HT, but the S^{35}-labeling in the heparin-extract was not correlated with the amount of heparin in the O and T cells: O cells had only 0·04 units of heparin/10^6 cells but showed almost as much S^{35}-label in the heparin extract as the T cells, which contained 0·14 units of heparin. The T cells, on the other hand, when compared with the X-2 cells, showed much less labeling than the heparin-value would lead one to expect. Ostensibly, then, there was no clear relationship between the amounts of heparin in a cell and its capacity to incorporate S^{35}-sulfate into heparin.

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When however, the radioactive heparin extract was chromatographed, a different picture emerged. The heparin was resolved into three radioactive spots: R_f 0·00, 0·76 and 0·94. The amount of radioactivity at R_f 0·00 was proportional to the amount of heparin in the sample and to the amounts of amines, especially 5-HT. Bovine heparin, chromatographed concomitantly, exhibited two mobile spots, none at R_f 0·00.

The relative proportions of the three heparins were the same in X-1 and X-2 cells; the O cells had preponderance of the R_f 0·76—heparin, and the T cells a relatively large amount of the immobile heparin. Since the relative amounts of these heparins remained the same within each cell line, measurements of total S^{35}-heparin within each cell line reflect the amount of heparin, a conclusion supported by the data of Table 3.

Metachromasia on the paper was shown by all three components, especially the immobile heparin. The amount of metachromasia at R_f 0·00 paralleled the amount of radioactivity there, and hence the total amount of heparin as measured by bioassay.

The heparins at R_f 0·76 and 0·94 were eluted with water and the heparin activity was measured by bioassay. The fast-moving component was inactive, but the R_f 0·76-heparin had slight anticoagulant activity. The immobile heparin could not be eluted with water and was eluted only partially with 0·1 M-Na_2HPO_4.

Intracellular distribution of 5-HT, histamine, and heparin in the solid mastocytoma. The apparent parallel between the levels of amines and the concentration of heparin suggested that the amines may be associated with heparin within the cell. X-1 cells were grown in the solid form (subcutaneously) in the mouse. Sixteen hours before removal and fractionation of the tumors, 1 mc of S^{35}-sulfate was injected. Histological examination of this tumor by Drs. A. Coulombre and R. Barrnett showed mast cells with variable amounts of granules, small cells with dense nuclei and no granules, connective tissue, fat, and blood vessels. The extracted heparin was determined by metachromasia with Azure A, and its S^{35}-labeling was measured. The bulk of the 5-HT, histamine and heparin was found in the particulate material, i.e., the fraction containing cell debris, nuclei and mitochondria (Table 5; column headed endogenous).

TABLE 5. PERCENTAGE DISTRIBUTION OF 5-HT, HISTAMINE AND HEPARIN IN THE SOLID X-1 MASTOCYTOMA

Fraction	Endogenous				Exogenous		
	5-HT	Histamine	Heparin Azure A	Heparin $S^{35}O_4$	5-HT	Histamine	Heparin Azure A
Nucleis and debris	24	33	50	51	23	10	16
Mitochondria	30	36	23	32	14	12	17
Microsomes	16	15	11	5	3	16	12
Supernatant	30	16	16	12	60	62	55

To rule out the possibility that these compounds are only adventitiously bound to particulate material following cell-disruption, histamine (1 μg/g of tissue), 5-HT (1 μg/g of tissue) and heparin (2·0 units/g of tissue) were added to the homogenate and the cells fractionated. It may be seen (Table 5; column headed exogenous) that the

major portion of these compounds, when added to the homogenate, remained in the supernatant fraction.

The increase in 5-HT, *histamine and heparin in culture.* To show more clearly the increase in 5-HT, histamine, and heparin when cells are grown in culture, the experiment presented in Fig. 10 was carried out. When taken from culture, X-1 cells had

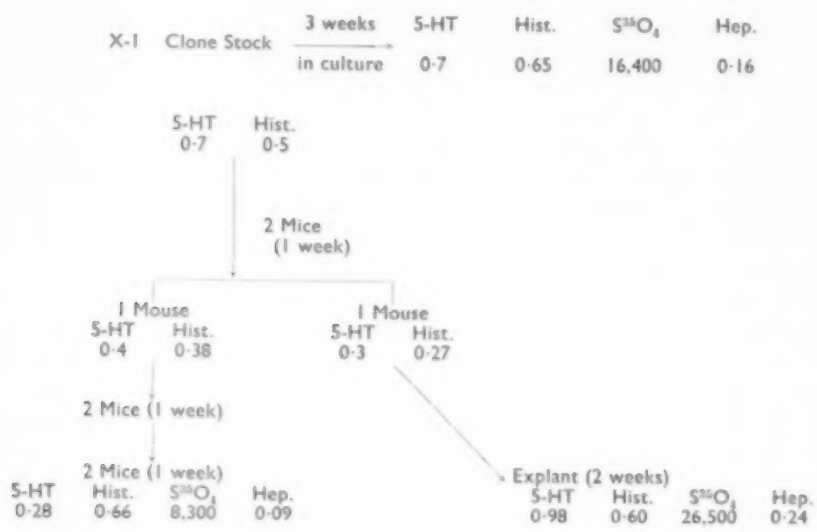

FIG. 10. Changes in the levels of 5-HT, Histamine, and Heparin produced in culture. All values are expressed as μg, units or counts/min. per 10^6 cells.

0·7 and 0·5 μg of 5-HT and histamine, respectively, per million cells. One aliquot of these cells, maintained in culture for three weeks, showed no significant change in the amount of 5-HT or histamine. Another aliquot of these cells was inoculated into mice and one week later, the cells were removed; 5-HT and histamine values in one of these mice had fallen to 0·4 μg and 0·38 μg, in another to 0·3 μg and 0·27 μg, respectively. When an explant of the tumor cells from one of the mice was made and allowed to grow in culture for two weeks, the 5-HT and histamine levels increased markedly (to 0·98 and 0·60 μg, respectively). When the same cells were passed through two pairs of mice for one-week intervals, the levels of 5-HT stayed low (0·28 μg); inexplicably, however, the histamine levels rose to 0·66 μg. With this single exception, the experiments showed that the intracellular levels of 5-HT and histamine increase in culture and decrease when the same cells are grown in the mouse.

Where possible, measurements of heparin were made by metachromasia and by determining the amount of S^{35}-sulfate incorporated into the heparin. It can be seen that the levels of heparin, by both methods, paralleled the amine levels, particularly that of 5-HT: the high concentration in the cells grown in culture fell after passage in the mouse and was restored when the cells were again placed in culture.

Nutritional and hormonal effects are two factors that may explain the increased concentration of these substances in culture. It appeared possible that in culture, the

concentration of the precursors and co-factors were more favorable for the synthesis of 5-HT, histamine, and heparin, not only because the levels of tryptophane and histidine may be higher in the culture medium than in plasma, but also because the number of metabolic pathways available for these amino acids and co-factors are fewer in isolated mast cells than in the whole animal. Accordingly, mice bearing X-1 cells were given seven daily intraperitoneal injections of one of the following: uridine (50 mg per day); glucuronate, glucosamine, tryptophane, or histidine, each 8 mg per day, and also the last three compounds in combination. Sixteen hr before harvesting the cells, S^{35}-sulfate was injected. None of the treated animals showed an increase in 5-HT, histamine or heparin or in S^{35}-sulfate incorporation. Similarly, X-2 cells grown for two weeks in a culture medium fortified by the addition to 1 l of medium of uridine (2·5 mg), glucosamine (2·5 mg), pyridoxal (0·05 mg), serine (5·0 mg), histidine (4·5 mg), tryptophane (1·5 mg), and 5-hydroxytryptophane (15 mg) showed no change in levels of 5-HT and histamine. That the size of the cell-inoculum did not influence the concentration of the amines in the cells was shown by the observation that 5-HT and histamine levels were the same whether cells were grown from a cell-inoculum of 3×10^4 cells/ml or 3×10^5 cells/ml. To test the possibility that dialysates of the horse serum needed for cell growth in culture might contain one or more factors that enhance the synthesis of the three amines, dialysates of 100 ml of horse serum were collected, lyophilized, restored to a volume of 25 ml, and 1 ml was injected daily for one week. There was no detectable increase in 5-HT, histamine, heparin, or S^{35}-sulfate-incorporation into heparin. Finally, it seemed possible that in the mouse certain hormones may inhibit the synthesis of the three substances. As cortisone has been shown to inhibit the formation of histamine[16] and the incorporation of S^{35}-sulfate into mast cells,[17] cortisone (1·25 mg) was injected daily for seven days into mice, some bearing X-1 cells, others X-2. Although cortisone reduced cell-growth to one-third of the normal rate, it did not alter the levels of 5-HT, histamine or heparin or the incorporation of S^{35}-sulfate into heparin, in either cell-line. A much more potent steroid, the acetonide of triamcinolone (i.e., 9-γ-fluoro-16-γ-hydroxyprednisolone; Kenalog.®), a gift of the Squibb Institute for Medical Research in a dose of 0·2 mg/day was administered for two days to another group of mice bearing X-1 cells; this therapy result in a 50 per cent reduction in cell growth, but had no effect on the four parameters mentioned.

DISCUSSION

It is clear that these neoplastic mast cells, like normal mast cells of the mouse and rat, not only store but produce 5-HT, histamine, and heparin. Normal rat mast cells *in vitro* decarboxylate 5-hydroxytryptophane[18] and histidine[19] while these neoplastic mast cells produce 5-HT from tryptophane when growing in culture and also as an homogenate of the mastocytoma.[8] Although direct evidence for the formation of histamine from histidine by these neoplastic cells is lacking, the presence of histamine in cells grown in a culture medium devoid of measurable amounts of histamine[4] strongly implies that they produce histamine from histidine, as has been shown in a canine mastocytoma.[20] While evidence that normal mast cells produce heparin is inferential, resting on the incorporation of sulfate[21, 22] and of glucose[23] into heparin in the intact animal, there is abundant evidence that neoplastic mast cells form heparin. As shown in this study the quantity of heparin in the culture medium is less

than 0·1 units and, thus it is far too low to account for the heparin-content of the cells. Moreover, the cell is unable to take up pre-formed heparin, though precursors of heparin viz., glucose, glucosamine, and sulfate, are incorporated into the sulfomucopolysaccharide by the cell in culture. Furthermore, slices of the tumor incorporate sulfate and glucose into heparin,[25] and homogenates[6, 24, 25] form 3′-phosphoadenosine-5′-phosphosulfate[26] and transfer its sulfate to heparin. Finally, the sulfate and glucosamine of heparin exchange at the same rate (Fig. 9), suggesting that the entire molecule of heparin is turning over, as in chondroitin sulfate,[27] rather than simply exchanging its sulfate and hexosamine. It follows from this that measurements of the incorporation into heparin of sulfate or glucosamine actually measure the synthesis of heparin, a conclusion supported by concomitant determinations of sulfate-uptake and heparin-concentration (e.g., Table 3). The turnover time of heparin of two-and-a-half days in these mast cells compares with three and one half days found in a dog liver[23] and contrasts, interestingly, with the ten-day turnover-time of chondroitin sulfate in rat skin.[27]

Failure to show the presence of heparin in the culture medium of actively growing and metabolizing cells suggests that mast cells, under these conditions, do not secrete heparin. There is no evidence that normal mast cells secrete heparin. [28]

Unlike glucose, glucosamine, and sulfate, glucuronate was not taken up by the mast cells. Its presence in heparin (see Walton[29]) is readily explained by oxidation of glucose to glucuronate.[30] Glucose was readily incorporated into heparin by both X-1 and X-2 cells at the same rate (Fig. 5), whereas X-1 cells in parallel experiments incorporated glucosamine (Fig. 6) more rapidly than X-2. The dilution of glucose-U-C^{14} by the high levels of non-labeled glucose in the medium may explain this discrepancy.

Compared with the near-diploid X-2 cells, the polyploid X-1 cells are less virulent. They usually, though not always, contain greater amounts of 5-HT, heparin, and histamine and have a greater capacity to take up and to incorporate into heparin both S^{35}-sulfate and glucosamine-1-C^{14}. The fact that X-1 cells are larger may appear to be an explanation for their having higher levels of these compounds, but this is difficult to reconcile with the observation that X-2 cells occasionally show the same or greater amounts of these compounds than X-1 cells (e.g., Table 1). It is likely that factors other than cell size influence these differences. A possible clue to some of the factors controlling the levels of these compounds may lie in the observation that higher levels are obtained in culture than in the mouse.

Attempts to increase the levels of heparin, 5-HT, and histamine in the mast cells by injecting their known precursors into mice bearing the tumor were unsuccessful. Similarly, increasing the levels of these precursors in the culture medium did not increase the levels of the amines. It would appear, then, that nutritional factors are not limiting the synthesis of these compounds. Another factor that could explain the lowered levels of these compounds in mast cells grown *in vivo* is the presence of hormones inhibiting synthesis or storage or acclerating loss. Thus, administration of cortisone to rats inhibits both sulfate-uptake by mast cells[17] and histamine-binding by tissues,[16] but in the mouse, histamine-binding by tissues was not influenced by cortisone.[17] Experiments presented here show that the administration of cortisone (and triamcinolone acetonide), in amounts that inhibited cell growth did not affect the concentration of heparin, histamine or 5-HT in neoplastic mast cells in the mouse. Other hormones are known to inhibit the synthesis of sulfomucopolysaccharides.[17]

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One of these hormonal factors could explain the higher levels found in culture and perhaps also the wide variation of the three substances found from one experiment to another. Whatever these factors may be, they need not necessarily influence directly the synthesis of 5-HT and histamine, but instead may control the levels of heparin which, in turn, may determine the limits of histamine and 5-HT which can be bound by the cell.

This suggestion arises in part from the observation that in every experiment in which levels of heparin and the amines were measured concomitantly, the concentration of amines, especially that of 5-HT, was found to be proportional to the concentration of heparin (Tables 2, 3, 4). Moreover, the three compounds were found to be associated intracellularly: in the experiments reported here, with the large granule fractions (Table 5) and in the experiments of Hagen *et al.*[31] with a specific granule fraction. The fact that the solid mastocytoma in the experiments described here contained cells other than mast cells (as contrasted with a population purely of mast cells when cells are grown in the ascitic form or in culture) does not invalidate the conclusion that the three compounds are associated in the same cellular fraction.

Riley and West[32] first noted the association of histamine and heparin in mast cells. This association may not be restricted to mast cells, for basophils contain histamine[33] and heparin (or a heparin-like compound),[34, 35] as do platelets of some species.[36, 37] The idea that histamine may be linked to heparin stems from (a) the proportionality of the two compounds in mast cells,[28, 38] (b) studies on histamine-release,[28, 38, 43] and (c) the observation that heparin and histamine can, *in vitro*, form a complex.[44–46] Although the possibility remains that the association of histamine with heparin is fortuitous or attributable to yet a third unknown factor, the data presented here are consistent with the idea that histamine may be linked to heparin in the mast cell.

In the test tube, histamine has an affinity for heparin which is about four times greater than that of 5-HT for heparin.[45] Yet, the amount of 5-HT in the mast cell is more closely proportional to the amount of heparin than is the amount of histamine. This relationship did not obtain in human platelets.[47] Instead, in these cells, the amount of 5-HT is proportional to the amount of adenosine triphosphate (ATP).[48] It is interesting to note that in buffered solutions, phosphate ions (and ATP) increased the affinity of histamine for heparin;[46] thus, it is possible that ATP may also be concerned in binding of amines in the mast cells. On the other hand, the mechanism of binding of amines may differ in different cells. Indeed, Mota and his associates[49] have noted that histamine is found in organs that lack mast cells and, although the amine may be bound to a sulfomucopolysaccharide like heparin, the fact that it is not released by compound 48/80 may indicate a different kind of binding. Similarly, reserpine releases histamine as well as 5-HT from both mast cells and rabbit platelets but has little or no effect on the histamine content of organs.[50] Moreover, Schayer[51] has shown that the capacity of a variety of organs to bind exogenous histamine differs from their content of histamine. Analogously, the platelet may bind 5-HT to a compound different from that by which it is held in the mast cell (or in the rabbit platelet).

The ease of extractability of histamine[41] and of 5-HT from tissues suggests that the amines are loosely bound, perhaps in an ionic type of linkage. Heparin has free sulfate and carboxyl groups as sites for such binding. If the sulfur-content of the heparin of mast cells is assumed to be 10 per cent, as in bovine heparin,[29] the number of sulfate groups in the mast cells are insufficient to account for the binding of amines (there are

about twice as many moles of amine as sulfate). There are several possible explanations for this discrepancy: (a) the carboxyl groups of heparin also bind amines; (b) the amines are held non-ionically; (c) only one of the amines is linked to heparin; (d) the heparin in the mastocytoma has a larger number of sulfate groups than bovine heparin or differs in some other way that would increase its affinity for amines.

That the mast-cell heparin is unusual is suggested by paper chromatography. The heparin-extract was resolved into three compounds only one of which was correlated with the cellular levels of the amines, especially of 5-HT (Table 4). This component was not present in chromatograms of bovine heparin. Thus, a unique heparin in the mast cells of the mouse may account for the capacity of these cells to bind large amounts of amines. If also present in the mast cells of the rat, it may explain the apparently anomalous presence of 5-HT in the mast cells of this species[18] as well as of the mouse.

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THE INTERACTION BETWEEN REVERSIBLE AND IRREVERSIBLE MONOAMINE OXIDASE INHIBITORS*

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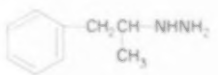
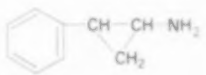
Abstract—The influence of the alkaloid harmine on the inhibition of monoamine oxidase (MAO) by β-phenyl*iso*propylhydrazine (PIH) and 2-phenyl*cyclo*propylamine (SKF-385) has been investigated. Rats pretreated with harmine were effectively protected from the long acting inhibitory effects of PIH, but not readily from those of SKF-385. The duration curves of the inhibition of MAO by PIH and SKF-385 in normal and harmine-pretreated animals were also compared. The animals treated with both harmine and PIH showed a short duration of action, typical of harmine alone, while those animals treated with both harmine and SKF-385 showed the usual SKF-385 duration curve. These findings suggest that PIH and harmine may act at the same site on the enzyme; however, the mechanisms of inhibition of MAO produced by PIH and SKF-385 may be quite different.

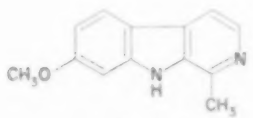
THE inhibition of the enzyme, monoamine oxidase (MAO), by a variety of hydrazine derivatives is in many ways similar to that produced by the organophosphorus compounds on cholinesterase (ChE). In both instances the inhibition is irreversible in nature, and when administered to intact animals both types of compounds lead to a prolonged inhibition of the respective enzymes.[1] The resulting pharmacological effects in these cases are presumably the result of an accumulation of the substrates in various areas of the body.



(JB – 516, PIH)



(SKF – 385)



Harmine

An interesting phenomenon associated with the inhibition of ChE is its apparent protection from irreversible inhibition by the organophosphorus compounds through prior treatment with physostigmine, a reversible ChE inhibitor. This effect was first described by Koster[2] and the mechanism demonstrated by Koelle.[3]

The current interest in MAO has made available a variety of inhibitors of this enzyme. Several of these agents have been employed in the present study. The chemical

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structures of these compounds are shown below; they are: harmine, β-phenyl*iso*propylhydrazine (JB-516, PIH), and 2-phenyl*cyclo*propylamine (SKF-385). Harmine is a potent, but short-acting, reversible inhibitor of MAO. PIH has been described as an irreversible and long-acting inhibitor,[4] similar to a number of other hydrazine compounds; SKF-385, a new type of MAO inhibitor,[5] also possesses a relatively long duration of action. The present paper is concerned with the influence of the short acting inhibitor, harmine, on the activity of the longer acting agents. This study was made to determine whether reversible inhibitors of MAO could protect the enzyme from the effects of the longer acting inhibitors.

METHODS

Male Sprague-Dawley rats weighing 175–250 g were used throughout these experiments. All drugs, with the exception of harmine, were injected subcutaneously. Harmine, in doses of from 5 mg/kg to 60 mg/kg, was administered intraperitoneally 35–40 min prior to either PIH or SKF–385. The doses of 1 mg/kg (PIH) and 2 mg/kg (SKF–385) were selected because preliminary studies showed them to produce about the same degree of inhibition 16–18 hr after their administration.

At various intervals after injection of the long-acting inhibitor the animals were sacrificed. The livers and brains were quickly removed, ground with Teflon homogenizers, and diluted to concentrations of 20 per cent and 33 per cent, respectively. One ml of the homogenate was incubated with serotonin creatinine sulfate (4 μmoles) and phosphate buffer (pH 7·0) for periods of 30 and 60 min, respectively. The resulting mixture was then assayed for residual serotonin, using the nitroso-naphthol colorimetric method, as described by Udenfriend *et al.*[6]

The extent of MAO-inhibition was calculated by comparing the amount of serotonin metabolized by the treated and control homogenates. All data are expressed in per cent of MAO-inhibition.

RESULTS

Fig. 1 indicates the influence of prior treatment of rats with 20 mg of harmine per kg, on the inhibitory effects of PIH and SKF-385 on MAO. These results were obtained 16–18 hr after the injections of the inhibitors. This time interval was used since the harmine effect was completely absent, while the normal effects of PIH or SKF-385 were still markedly present. It is apparent that in those animals pretreated with harmine, PIH did not exert its effect. Control PIH animals showed an average of 86 per cent inhibition in brain and 65 per cent inhibition in liver, while tissues from harmine-pretreated animals showed essentially no inhibition. With SKF-385, there was no apparent difference between harmine-pretreated and the control animals. The MAO in both liver and brain, in harmine-pretreated animals, as well as in control animals, responded similarly to SKF-385; the inhibition observed was about 70 per cent inhibition in brain and 57 per cent in liver. Each of the bars in Fig. 1 is the mean value from five to ten animals.

When the harmine dose was lowered the degree of antagonism of the effect of PIH was decreased. As indicated in Table 1, doses of 10 and 5 mg of harmine per kg had progressively diminishing effects on the inhibition of MAO by PIH. However, even with the lowest dose used (5 mg/kg), harmine still exhibited a considerable antagonism of PIH. In contrast to PIH the antagonism of the effect of SKF-385 on MAO was

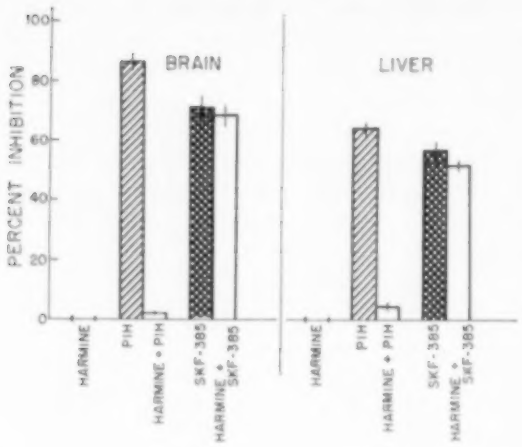

FIG. 1. The influence of harmine-pretreatment (20 mg/kg) on inhibition of brain and liver MAO by PIH and SKF-385. These values of MAO inhibition were obtained 16–18 hr after injection of the indicated agents. Each bar represents the mean of 5–10 experiments. Vertical lines indicate the S.E.M.

difficult to demonstrate even with toxic doses of harmine. Doses of 40 and 60 mg/kg were used, and at the highest dose the animals showed marked tremors and motor incoordination; four of the eight animals treated in this manner succumbed within 12 hr. Even with these high doses of harmine the effects of SKF-385 were not altered significantly in the liver; in the brain, however, some antagonism was shown, as can be seen in Table 1.

TABLE 1. THE EFFECT OF HARMINE ON THE INHIBITION OF MAO BY PIH AND SKF–385

Pretreatment with Harmine (mg/kg)	I percent MAO inhibition by PHH[1] Brain	Liver
0	86 ± 3·0 (5)	64 ± 2·4 (6)
5	26 ± 5·9 (4)	27 ± 3·6 (3)
10	11 ± 2·0 (4)	13 ± 1·6 (4)
20	2 ± 0·1 (5)	5 ± 2·6 (6)
	by SKF–385	
0	71 ± 3·1 (9)	57 ± 3·0 (10)
20	68 ± 3·5 (5)	51 ± 2·4 (6)
40	53 ± 4·8 (6)	54 ± 4·4 (6)
60	48 ± 5·5 (4)	49 ± 3·9 (4)

The figures listed above are the mean values of the percent of MAO inhibition ± the S.E.M., the standard error of the means. Figures in parentheses indicate the number of experiments carried out at each dose level.

Figs. 2 and 3 demonstrate the duration of MAO-inhibition produced by these three compounds on the MAO of brain, and its modification by pretreatment with harmine. In both of these figures each point represents the average values from two animals. Harmine itself had a short duration of action. With the doses used in these experiments

(20 mg/kg), essentially complete inhibition of the MAO of brain was produced within 15 to 30 min after administration. Within 4 hr at least eighty per cent of the enzyme activity had returned, and after 12 hr recovery was complete. PIH, on the other hand, exerted a prolonged inhibition of MAO, as can be seen in Fig. 2. Even 48 hr after its

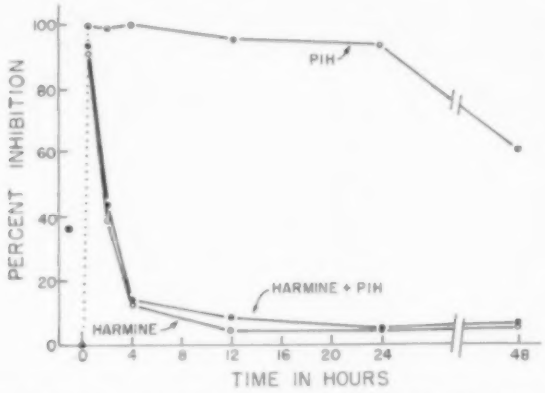

FIG. 2. The duration of action of harmine (20 mg/kg) and PIH (1 mg/kg) on brain MAO, and the effect of harmine-pretreatment on the duration of action of PIH. Drugs were administered at zero time. Each point represents the average values obtained from two animals.

administration there was still about a 60 per cent blockade of the brain enzyme. In the harmine pretreated animal, however, the long duration of action of PIH was completely absent. Instead of a prolonged action, PIH in these cases closely approximated the duration curve typical of harmine alone, which indicates that the latter agent had prevented the effects of PIH from being exerted.

In Fig. 3, SKF-385 is shown to exert a relatively long duration of action. Like PIH and harmine its onset is rapid, and there is a gradual decrease in its inhibitory effect over 24 and 48 hr. At the end of 48 hr there was still a 40–45 per cent inhibition of brain MAO. In harmine-pretreated animals the course of the inhibition by SKF-385

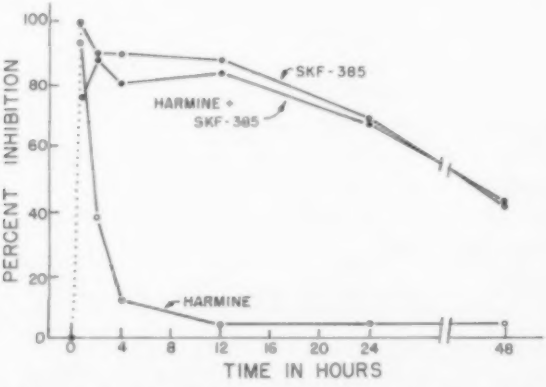

FIG. 3. The duration of action of harmine (20 mg/kg) and SKF-385 (2 mg/kg) on brain MAO, and the effect of harmine on the duration of action of SKF-385. Drugs were administered at zero-time. Each point represents the average values obtained from two animals.

appeared to be unaffected. In both control and harmine-pretreated animals the inhibition induced by SKF-385 followed approximately the same time course, suggesting that harmine in this dose did not interfere with the inhibitory action of SKF-385.

DISCUSSION

The results obtained in these experiments indicate that it is possible to protect the enzyme MAO from the long-acting inhibitory effects of PIH by first treating the animal with harmine, a short-acting reversible inhibitor. Similar indirect evidence also has been presented by Pletscher *et al*,[7,8] who found that the rise in brain levels of serotonin and norepinephrine resulting from the administration of iproniazid or glutamic acid-α-isopropylhydrazide could be reduced by prior treatment with harmaline. They attributed this effect to the inability of the hydrazine compounds to attack MAO which had been pretreated with harmaline, a circumstance which prevented the accumulation of the substrates of MAO in the brain. The present study confirms this hypothesis by direct examination of the enzyme system and has also demonstrated that even the extremely potent hydrazine derivatives, such as PIH, are effectively antagonized by pretreatment with harmine.

It is possible that the antagonism described here is the result of both drugs acting at the same site on the enzyme surface, and that the prior treatment with harmine prevents the occupation of the active site by PIH. A similar hypothesis has been offered to explain the antagonism of the DFP effects by physostigmine.[3] This phenomenon is seen only when the harmine is administered before the irreversible agent. If PIH be given before the shorter acting harmine, then the antagonism is no longer present, but the usual long acting effects of the hydrazines are produced. It would appear, therefore, that once the enzyme is irreversibly inhibited by PIH, harmine is no longer capable of attaching to the active site of the enzyme. This seems likely, since the inhibition of MAO by the hydrazines is irreversible,[1,9] while that produced by harmine is reversed quite readily.[10]

The inability of harmine-pretreatment to antagonize the long acting MAO-blocking actions of SKF-385, in the same manner as with PIH, suggests that its mechanism of action might differ from that of the hydrazine derivatives. No actual reports have appeared to indicate whether SKF-385 is a reversible or irreversible inhibitor, although structurally it resembles the reversible type of agent, such as amphetamine and ephedrine. Its relatively prolonged action, as demonstrated in these experiments, more closely resembles the irreversible type of inhibitor. Which ever the case may be, it appears that SKF-385 may be displacing harmine and itself occupying the active site. This is a possibility since some antagonism of the effect of SKF-385 was seen in brains of animals pretreated with very high doses of harmine. Thus, SKF-385 appears to have a very high affinity for the MAO enzyme. Some of these problems are under investigation at the present time.

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IN VIVO REACTIVATION BY PAM OF BRAIN CHOLINESTERASE INHIBITED BY PARAOXON*

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Abstract—Pyridine-2-aldoxime methiodide (PAM) injected in amounts of from 25 to 150 mg/kg from 0·5 to 1·5 hr after 0·15 mg/kg paraoxon caused a significant increase in the cholinesterase levels of cerebral cortex, pons, cerebellum, region of area postrema in medulla, and the remainder of the medulla of rabbits killed 3 hr after the injection of paraoxon. Reactivation seemed most marked in the pons, region of area postrema and the remainder of the medulla. Similar results were obtained when rabbits were killed 24 hr after paraoxon, and there appeared to be no difference whether the first dose of PAM was given 1 or 5 hr after the inhibitor. A chloroform extraction procedure was used which appears capable of removing at least some of the extracellular paraoxon present in the brain tissue and uncombined with the enzyme at the time of sacrifice of the rabbits. It is concluded that sufficient amounts of PAM are able to penetrate into the brain to cause marked reactivation of inhibited cholinesterase. Possible reasons are discussed why other workers have not observed *in vivo* reactivation of brain cholinesterase by PAM.

INTRODUCTION

SOME organophosphorus compounds are powerful potential chemical warfare agents (the so-called "nerve gases"), others are widely used as insecticides. These compounds are potent irreversible inhibitors of ester splitting enzymes in general, but their lethal action must be attributed to the inhibition of acetylcholinesterase, the enzyme which hydrolyses acetylcholine and is vital for nerve activity. Analysis of the molecular forces at the active site of acetylcholinesterase and the mechanism of the hydrolytic process has revealed the mode of action of these poisons: a nucleophilic group at the active site, acetylated in the normal process, is phosphorylated; this process is not readily reversible and thereby the enzyme is inactivated.[1, 2] It may, however, be reactivated by nucleophilic compounds which in a displacement reaction remove the phosphoryl group from the enzyme. In systematic studies of the forces acting in the enzyme surface, a powerful reactivator of the phosphorylated enzyme has been developed (pyridine-2-aldoxime methiodide-PAM).[3] The compound proved to be an effective antidote against some of the nerve gases and insecticides, especially in combination with atropine.[4–10]

Paralysis of respiration seems to be the main cause of death in all cases of alkylphosphate poisoning tested. Although the respiratory failure produced by some compounds appears to be predominantly the result of a paralysis of the diaphragm, central effects are in many instances an important additional factor. Reactivation of acetylcholinesterase in the diaphragm of mice treated with PAM after exposure to an

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alkylphosphate was demonstrated by Kewitz.[13] Only slight reactivation of enzyme activity by PAM was found in the brain of mice exposed to paraoxon and no reactivation after injection of DFP (di*iso*propyl fluorphosphate).[7] Other observers were unable to find any reactivation in brain.[10–12] It was therefore assumed that PAM, being a quaternary ammonium ion, is unable to pass through the blood-brain barrier in sufficient amounts to produce reactivation of the enzyme. On the other hand, Bovet and Longo found a definite effect of PAM on the electroencephalogram of rabbits exposed to sarin.[14] This observation suggests a central action of the antidote.

It appeared of both practical importance and of theoretical interest to find an unequivocal answer to the problem of whether or not PAM is able to penetrate the blood-brain barrier and repair the chemical lesion in the brain of alkylphosphate poisoned animals by reactivating the enzyme activity either in the entire brain or in some vital areas. This paper records the results of an investigation of this problem.

MATERIALS AND METHODS

All injections were given intravenously into adult male albino rabbits weighing from 2·5 to 3·0 kg. Diethyl-*p*-nitrophenyl phosphate (paraoxon) was used as a representative anticholinesterase and all dilutions for injections were made with saline. PAM was dissolved in distilled water. Sufficient sodium hydroxide was added to give an unbuffered solution of pH 8, which when injected in a dose of 1–2 ml would not alter the blood pH.

At specified intervals of time after poisoning with paraoxon, the rabbits were sacrificed by the intravenous injection of air, their brains removed and dissected into the areas used for determination of cholinesterase activity. All brain areas unless otherwise specified were extracted with chloroform by a procedure modified from that described by Kewitz.[13] The samples were sliced by hand, weighed and frozen in dry ice. They were then lyophilized and each sample placed in 15 ml of — 60 °C chloroform for 0·5 hr during which time they were stirred, and the extractant replaced by fresh chloroform. They were filtered in funnels surrounded by dry ice, rinsed with ether and dried. The tissues were then homogenized in Krebs phosphate media;[15] the suspension containing 5–10 mg wet wt/ml, depending on the activity expected, except for samples from the region of the area postrema where due to the small amount of tissue obtainable the final concentration was 2·5–3·5 mg/ml. Acetylcholine chloride at a concentration of 5×10^{-2} M was used as the substrate, and all tissues were incubated while shaking in a water bath, at 38 ° for 2 hr. Acetylcholine was employed as the substrate instead of substrates more specific for different types of cholinesterase, since it is known that the great majority of cholinesterase in the brain is of the specific type.[16] In addition, low concentrations of PAM reactivate almost exclusively acetylcholinesterase due to molecular complementarity and not the non-specific esterases. Since probably only small amounts of PAM will penetrate into the brain, it is highly unlikely that any non-specific esterases would be reactivated. Cholinesterase activity was determined at the 1 and 2 hr periods of incubation by the Hestrin[17] colorimetric procedure.

RESULTS

A procedure often used for determining cholinesterase activity of tissues from animals previously exposed to alkylphosphates or other inhibitors of cholinesterase

has been that of preparing a homogenized suspension immediately after removal of the tissue. This may not, however, indicate the true cholinesterase activity of the cells at the time of death or sacrifice of the animals, since a portion of the alkylphosphate may have been localized outside of the cell, and excess inhibition may thereby be produced during homogenization. This problem was pointed out by Nachmansohn and Feld.[18] Recently Kewitz and Nachmansohn have attempted to avoid these difficulties through a procedure by which most of the alkylphosphate in the extracellular spaces seemed to have been removed.[7, 13]

Before determining whether PAM can reactivate inhibited brain cholinesterase it was necesssry to devise an extraction procedure which would remove with the greatest possible efficiency the excess alkylphosphate uncombined with cellular cholinesterase. The precedure found most satisfactory is described in the methods section. Other techniques employing acetone, ether and methanol as extractants for varied lengths of time were tried. None of these procedures were found to be satisfactory either because the loss of enzyme activity compared with the control was too great or because the extraction of uncombined alkylphosphate was inefficient. It was found that control cerebral cortical tissue extracted with chloroform had 75 $\pm$ 2·7 per cent of the control unextracted values. This 25 per cent decrease in activity is probably partly due to denaturation and partly to unavoidable losses involved in the transfer of the tissue slices. Unextracted cerebral cortical tissue from rabbits treated with 0·2 mg/kg paraoxon and sacrificed 2 hr later had 5·6 $\pm$ 0·12 per cent of the normal cholinesterase levels. When the tissue was extracted with chloroform, all other conditions being equal, the activity was 11·5 $\pm$ 0·41 per cent uncorrected for the 25 per cent loss. Each of these above values are the means $\pm$ standard error based upon determinations on three rabbits. No correction for loss in control activity was applied to the data to be presented, since the extraction procedure was uniformly used in all experiments including the controls. The approximately twofold increase of cerebral cortical enzyme levels due to chloroform extraction is even more significant when one considers the loss due to extraction. It was observed that when rabbits were killed 24 hr after paraoxon, the extraction procedure caused no increase in the cholinesterase values. Apparently by this time all free paraoxon had been inactivated or removed. For uniformity, however, the tissues obtained from rabbits killed 24 hr after paraoxon were extracted in the usual manner.

Before testing the enzyme activity in brain tissues of paraoxon treated rabbits, the cholinesterase levels in various areas of the brain of normal animals were determined. The mean activities based upon determinations from three rabbits are as follows: cerebral cortex 265 $\pm$ 5·0, medulla 407 $\pm$ 17·6, pons 460 $\pm$ 25·2, cerebellum 403 $\pm$ 43·4 and region of the area postrema 530 $\pm$ 5·0 μmoles acetylcholine hydrolysed per g per hr. In order to test whether PAM reactivates brain cholinesterase, rabbits were killed either 3 or 24 hr after paraoxon or paraoxon plus PAM. The dose of paraoxon used (0·15 mg/kg) killed about 20 per cent of the rabbits; on these no determinations were made. In the 3 hr experiments PAM was injected either in a single dose of 100 mg/kg 1 hr after paraoxon, or in three doses of 40 mg/kg each at 0·5, 1 and 2 hr after paraoxon. Since there was no significant difference between these two procedures the results were combined. In Fig. 1 the results are presented in per cent of the mean control values based upon determinations from three or four rabbits for each brain area. In the 24 hr experiments PAM was injected in five doses of

50 mg/kg each over a period from 1 to 8 hr after injection of paraoxon. Fig. 2 presents the results with each percentage being based on determinations from four or five rabbits.

The cholinesterase activity of all brain areas tested, from the 3 and 24 hr experiments, are significantly ($P < 0{\cdot}01$ in all cases) greater in the rabbits that received PAM than in those which did not receive PAM. Examination of the data also indicates that the pons, the region of area postrema and the remainder of the medulla show greater reactivation by PAM than do the cerebral cortex and cerebellum, especially at the 3 hr interval of time. A single experiment with the entire brain minus the five areas

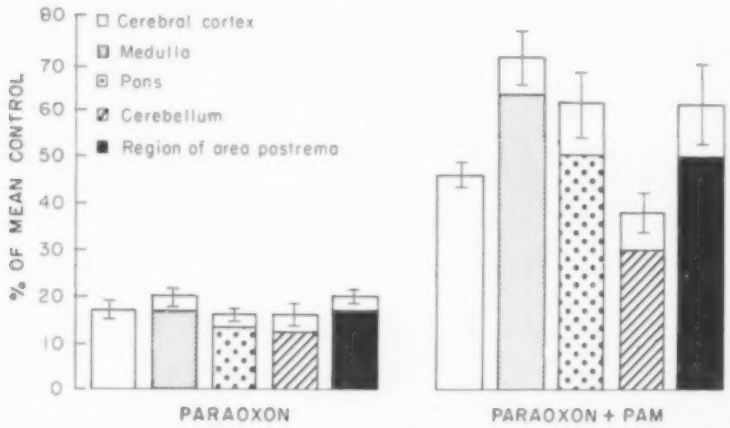

FIG. 1. Reactivation of inhibited cholinesterase by PAM. All rabbits received 0·15 mg/kg paraoxon 3 hr before sacrifice. PAM 100–150 mg/kg injected 0·5–2 hr after paraoxon. Lines at top of bars indicate ± 1 standard error.

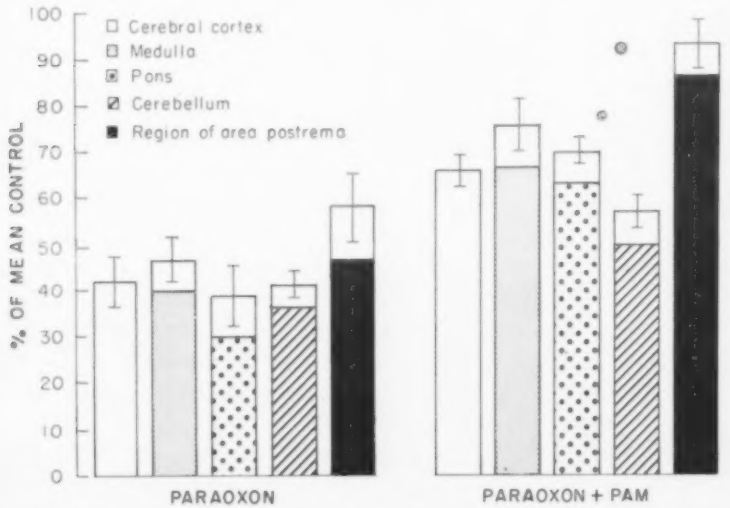

FIG. 2. Reactivation of inhibited cholinesterase by PAM. All rabbits received 0·15 mg/kg paraoxon 24 hr before sacrifice. Pam 250 mg/kg injected in five divided doses 1–8 hr after paraoxon. Lines at top of bars indicate ± 1 standard error.

listed in Fig. 1 indicates that PAM reactivates this cholinesterase to about the same extent as in cerebral cortex and cerebellum.

Since antidotal effects of PAM have been obtained with smaller doses than 100 mg/kg the reactivation of brain cholinesterase by 25 mg/kg PAM was compared in one experiment to that by 100 mg/kg PAM. The results are presented in Fig. 3. The

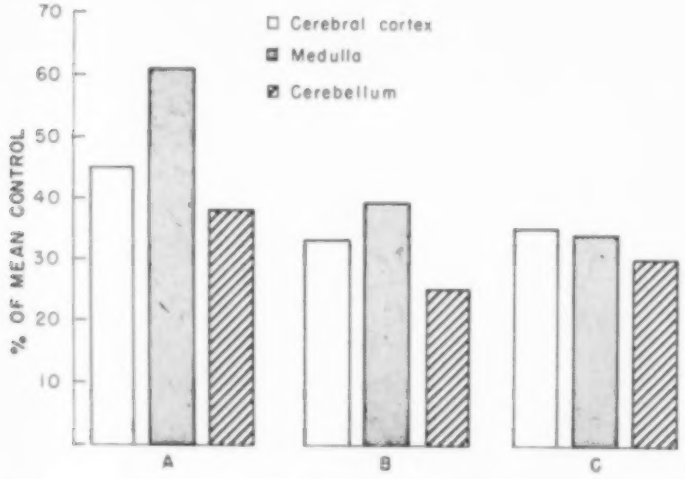

FIG. 3. Effect of alteration of dosage and time of administration of PAM upon reactivation of inhibited cholinesterase. All rabbits received 0·15 mg/kg paraoxon 3 hr before sacrifice. A, PAM 100 mg/kg injected 1 hr after paraoxon. B, PAM 25 mg/kg injected 1 hr after paraoxon. C, PAM 100 mg/kg injected 2 hr 45 min after paraoxon (15 min prior to sacrifice).

enzyme levels are not increased as much as with 100 mg/kg PAM but still appear significantly greater than with paraoxon alone (Fig. 1).

The question may be raised whether the reactivation obtained was an artifact due to *in vitro* reactivation of inhibited cholinesterase by free PAM present in the blood vessels of the brain at the time of homogenization. The 1 to 2 hr interval between the administration of PAM and the sacrifice of the animal in the 3 hr experiments was, however, probably sufficient for removal of most of the excess PAM. In the 24 hr experiments about a 16 hr interval was present between the last dose of PAM and sacrifice of the animal, which was undoubtedly sufficient for removal of uncombined PAM. To investigate this problem further an experiment was performed in which 100 mg/kg PAM was injected 15 min before the sacrifice of rabbits which had received paraoxon. These conditions would be expected to be favourable for any *in vitro* reactivation. As seen in Fig. 3 however the reactivation was actually less than when PAM was given 2 hr before death. The reason may be that PAM had not had sufficient time to penetrate into the brain to its maximum extent.

In order to minimize the possibility of a direct reaction between PAM and paraoxon in peripheral tissues, thereby decreasing the concentration of inhibitor which penetrates into the brain, PAM was injected in five doses of 50 mg/kg each over a time interval from 5 to 12 hr after the injection of paraoxon. The rabbits were sacrificed 24 hr after the injection of paraoxon. Each bar in Fig. 4 is the mean determination

from two rabbits. Under these conditions little free paraoxon would be expected to be present peripherally at the time when the first dose of PAM was given. The reactivation obtained was very similar to that observed in Fig. 2 where PAM was injected from 1 to 8 hr after paraoxon.

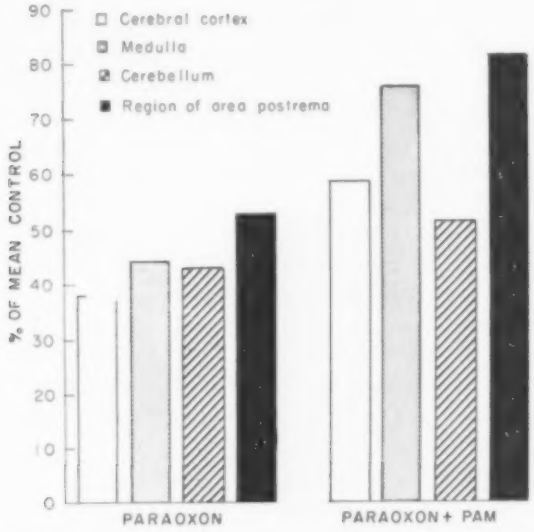

FIG. 4. Effect of delayed administration of PAM upon reactivation of inhibited cholinesterase. All rabbits received 0·15 mg/kg paraoxon 24 hr before sacrifice. PAM 250 mg/kg injected in 5 divided doses 5–12 hr after paraoxon.

DISCUSSION

Some reports indicate that *in vivo* PAM does not reactivate brain cholinesterase inhibited by alkylphosphates.[10–12] Since PAM *in vitro* can dephosphorylate brain cholinesterase inhibited by alkylphosphates[7, 11, 19] the lack of *in vivo* effect was explained as being due to the inability of PAM to penetrate into the brain. The reasoning was based on the fact that PAM is a quaternary ammonium ion; this type of compound is thought to penetrate the blood–brain barrier only slightly.[20, 21] The brain, however, cannot be considered as a single compartment through which drugs distribute themselves uniformly. It has been shown that certain areas of the brain such as the neurohypophysis, intercolumnar tubercles and area postrema apparently lack a well-developed blood–brain battier.[22, 23] This was one of the reasons for selecting among other areas the region of the area postrema in the present study. Differences in the penetration of PAM across the blood–brain barrier may explain why the pons, region of area postrema, and remainder of medulla showed greater reactivation of cholinesterase than did the cerebral cortex and cerebellum. Since many of the most vital central control centres, such as the respiratory centre are located in the medulla, near the area postrema, it is perhaps very important that the greatest reactivation was also obtained in these areas. The lack of reactivation reported in other investigations[10, 12] may be due to the omission of efficient extraction procedures. Since these workers used inhibitors which *in vitro* are reactivated by PAM, it does not appear likely that the reactivation observed here is due to the use of a different

inhibitor. Certain drugs may have different actions dependent upon the species as was shown for the metabolism of hexobarbital,[24] and since the other workers used rats and mice whereas we used rabbits, it is possible that there is a species difference in the penetration of PAM into the brain.

Our extraction procedure is probably far from 100 per cent effective in extracting uncombined inhibitor, as indicated by the fact that if rabbits were sacrificed 0·5 or 1 hr after high doses of paraoxon the cholinesterase values were lower than those in animals sacrificed after 3 hr and there was in the former experiments little difference between extracted or unextracted tissues (unpublished observations). The excess of uncombined paraoxon was apparently so great that the chloroform extraction was unable to remove enough paraoxon to prevent the inactivation of cholinesterase during homogenization.

In rabbits that received paraoxon alone there is a significant difference in the enzyme levels between those sacrificed at 3 hr and those sacrificed at 24 hr. This is probably because the extraction procedure is not perfect, and therefore the 3 hr values too low. Some spontaneous reactivation of inhibited cholinesterase and some synthesis of new enzyme also may occur *in vivo* in the course of 24 hr, but based upon the report of Oberst and Christensen[25] on the time required for spontaneous recovery of cerebral cholinesterase it appears unlikely that these two processes are adequate to explain the entire increase in cholinesterase activity.

When the first dose of PAM was given 5 hr after paraoxon it still was effective in causing an increase in brain cholinesterase levels although most of the extracellular paraoxon must have been removed by that time. It is also known that *in vitro* PAM reacts very slowly with paraoxon.[5] Based upon these two facts it appears safe to assume that the greater activity of brain cholinesterase observed when PAM is administered is not due to a direct reaction of PAM with paraoxon peripherally thereby decreasing the concentration of paraoxon which penetrates into the brain.

The brains were removed as fast as possible after death in order to avoid the breakdown of the blood–brain barrier. At most from 4 to 5 min were required to remove the brain. It is doubtful that in such a short period of time there is a sufficient breakdown of the blood–brain barrier to allow enough PAM to penetrate and in addition to reactivate the enzyme. Although the possibility of some breakdown of the barrier cannot be ruled out, it cannot be the only or most important factor responsible for the observed action of PAM, as indicated by Fig. 3. PAM, injected 2 hr and 45 min after paraoxon but 15 min prior to sacrifice, resulted in less reactivation than PAM, injected 1 hr after paraoxon but 2 hr prior to sacrifice. If there was a breakdown of the blood–brain barrier after death, we would expect greater reactivation since more PAM is present in the blood vessels of the brain when the rabbits are killed 15 min after the injection of PAM than when killed 2 hr after its injection.

Many quaternary ammonoium ions are toxic and, *in vivo*, can only be used in small doses. Thus, when small doses of such compounds are administered, the barrier to their penetration into the brain usually effectively prevents entry, with the result that only very small amounts or none are found in the brain. Because of the relatively low toxicity of PAM high doses can be used. With a dose of 100 mg/kg intravenously one would expect, assuming uniform distribution and neglecting inactivation, a concentration of about 10^{-4} M in the tissues including the blood vessels which initially will probably have a higher concentration. Even assuming a blood–brain ratio for

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PAM of 100 to 1 this would still provide a concentration inside the brain tissue of 10^{-6} M which is probably sufficient for reactivation of a significant percentage of inhibited cholinesterase.[3, 26] With doses of PAM lower than 25 mg/kg, which was the lowest dose used in these experiments, it is possible that no *in vivo* reactivation of cholinesterase would be observed, although undoubtedly even with small doses some PAM does penetrate into the central nervous system. It appears that central reactivation of cholinesterase, especially in the vital areas, such as the respiratory centres is an important factor in the antidotal effect which PAM exerts against paraoxon and possibly other anticholinesterases.

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THE EFFECT OF PYRROLIZIDINE ALKALOIDS ON LIVER ENZYME SYSTEMS

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Abstract—The effect of the pyrrolizidine alkaloids, lasiocarpine and heliotrine, on the activity *in vitro* of mitochondrial enzyme systems has been studied. The alkaloids inhibit enzyme systems which require pyridine nucleotides for electron transfer, do not affect cytochrome oxidase activity and stimulate succinoxidase activity when mitochondria are suspended in 0·25 M sucrose. Neither alkaloid influenced the activity of L-malic dehydrogenase or succinoxidase in water-disrupted mitochondria. Similar results with supinine, sarracine, monocrotaline and platyphylline indicate that the effect on mitochondrial metabolism may be common to pyrrolizidine alkaloids generally.

The effect on mitochondrial metabolism is associated with the nitrogen atom of the cyclic base of the alkaloids, as heliotridine inhibits like the parent alkaloid and the N-oxides do not influence mitochondrial oxidations.

Inhibition of mitochondrial enzyme systems is largely prevented by added DPN, and, to a lesser degree, by EDTA. The alkaloids increase mitochondrial permeability under oxidizing conditions and accelerate the loss of pyridine nucleotides thus inactivating dependent enzyme systems and stimulating succinoxidase activity.

The relevance of the mode of action *in vitro* to the acute hepatoxic effects of the pyrrolizidine alkaloids *in vivo* is discussed.

INTRODUCTION

BULL *et al.*[1] have shown that ingestion by sheep of the plant, *Heliotropium europaeum*, may produce disease of the liver. Pyrrolizidine alkaloids isolated from *H. europaeum*[2–4] were found to be the active principles;[5–7] two major alkaloids, heliotrine and lasiocarpine, possess considerable biological activity. Small doses of heliotrine or lasiocarpine when administered to rats cause chronic liver damage;[6] larger doses cause liver necrosis[5] or rapid death probably due to blockage of impulse transmission at neuromuscular junctions.[8] Heliotrine has been found to be mutagenic[9] and lasiocarpine has been reported to be carcinogenic.[10]

Biochemical disturbances associated with the acute hepatotoxic action of heliotrine have been investigated by Christie.[11] With rats receiving one large dose of heliotrine there was inactivation of enzyme systems requiring pyridine nucleotides for electron transfer and, consequently inactivation of tricarboxylic-acid-cycle activity just before hepatic necrosis became histologically demonstrable.

In the present paper, studies of the effects of pyrrolizidine alkaloids *in vitro* on enzyme preparations from the liver of the normal rat are reported.

* Abbreviations used in this paper: AMP, adenosine-5-monophosphate: DPN, diphosphopyridine nucleotide; GSH, reduced glutathione; EDTA, ethylenediaminetetra-acetic acid; HDP, fructose-1-6-diphosphate.

EXPERIMENTAL

Enzyme preparations. Rats were killed by stunning and decapitation. The liver was removed rapidly and immersed in ice-cold 0·25 M sucrose. Homogenates were prepared in 0·25 M sucrose and fractionated by differential centrifuging at 0–1°.[12] Mitochondria were resuspended in 0·25 M sucrose, except where otherwise indicated.

Reagents. Pyrrolizidine alkaloids were obtained as gifts. The alkaloids were dissolved in an equivalent amount of 1N HCl. AMP, DPN, nicotinamide, GSH, cytochrome *c*, citrate, α-oxoglutarate, L-glutamate, succinate, L-malate, octanoate, oxalo-acetate, L-ascorbic acid, EDTA, ethanol and inorganic reagents were obtained commercially.

Succinic acid was purified by recrystallizing three times from hot water; then dissolved, neutralized with 1N NaOH and precipitated as sodium succinate with ethanol. Sodium succinate was redissolved and crystallized from 80% ethanol. Octanoic acid was purified by distillation *in vacuo*. L-Ascorbic acid and α-oxoglutaric acid were dissolved and neutralized with NaOH just before use. AMP and other substrates were neutralized and stored frozen as sodium salts for 3–4 weeks.

Methods. O_2 uptake and CO_2 evolution were measured by Warburg manometers. Spectrophotometric readings were taken with a Beckman model DU instrument.

RESULTS

Experiments were done in duplicate and repeated at least once. Results are expressed as the means of relevant experiments.

(a) *Effect of lasiocarpine and of heliotrine on oxidative metabolism*

As shown in Tables 1 and 2 both lasiocarpine and heliotrine inhibited oxidations by rat liver mitochondria. The pattern of inhibition with either alkaloid was the same, but lasiocarpine proved inhibitory in lower concentrations than heliotrine. Mitochondrial oxidation of those substrates requiring pyridine nucleotides for electron transfer was inhibited, whereas succinate oxidation was appreciably stimulated. With citrate, oxidation was less inhibited than with all other substrates except succinate. In experiments where alkaloids were added from side-arm compartments of Warburg flasks, inhibition developed rapidly and reached full expression within 15 min.

TABLE 1. EFFECT OF LASIOCARPINE ON OXIDATIONS BY LIVER MITOCHONDRIA

Additions	Oxygen uptake (μl/30 mins)					
	Control	Lasiocarpine				
		33 mM	16·7 mM	6·7 mM	3·3 mM	1·67 mM
Citrate 10 mM	33	3	14	24	29	32
α-Oxoglutarate 10 mM	43	0	3	3	35	40
L-Glutamate 10 mM	30	3	3	2	16	26
Succinate 10 mM	67	91	103	99	83	—
L-Malate 10 mM	42	3	0	4	13	33

System: AMP 1 mM; $MgSO_4$ 6·7 mM; KCl 25 mM; NaK phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; mitochondria equivalent to 50 mg of fresh rat liver added in 0·25 ml of 0·25 M sucrose; water to 1·5 ml final volume; 0·1 ml of 20% KOH was placed in centre well to absorb CO_2; gas phase, air; temperature, 38 °C; equilibration period 10 min.

Oxidative metabolism by liver homogenates was inhibited by lasiocarpine and heliotrine in a manner similar to inhibition of mitochondrial oxidation. The oxidation of octanoate was found to be inhibited by either alkaloid when assayed with homogenate preparations. Isolated mitochondria were not used.

The activity of L-malic dehydrogenase was assessed in liver homogenates which were subjected to osmotic disruption by preparation in water instead of in 0·25 M sucrose.

TABLE 2. EFFECT OF HELIOTRINE ON OXIDATIONS BY LIVER MITOCHONDRIA

Additions	Oxygen uptake (μl/30 min)					
	Control	Heliotrine				
		67 mM	50 mM	33 mM	16·7 mM	6·7 mM
Citrate 10 mM	41	—	34	45	48	55
α-Oxoglutarate 10 mM	43	—	9	29	43	46
L-Glutamate 10 mM	36	—	5	28	37	32
Succinate 10 mM	91	112	113	111	112	—
L-Malate 10 mM	36	—	2	9	22	34
Oxaloacetate 10 mM + pyruvate 0·67 mM	55	—	4	3	33	48
Pyruvate 10 mM + L-malate 0·67 mM	35	—	10	21	26	33

System: as in Table 1.

The enzyme was assayed in the presence of added DPN. The reaction mixture contained L-glutamate to remove, by transamination, oxaloacetate, which inhibits the oxidation of L-malate. Neither 33 mM lasiocarpine nor 50 mM heliotrine had any effect on L-malate oxidation under these conditions, showing that these alkaoloids do not directly inhibit L-malic dehydrogenase.

Neither 33 mM lasiocarpine nor 50 mM heliotrine affected cytochrome oxidase activity of liver homogenates.

N-Oxides of both alkaloids were found to be without effect on mitochondrial or homogenate oxidations at the highest concentrations shown for the parent alkaloid in Tables 1 and 2. Neither substance inhibited nucleotide-dependent enzymes or stimulated the succinoxidase system.

(b) *Effect of lasiocarpine and heliotrine on anaerobic glycolysis*

Anaerobic glycolysis of HDP by liver homogenates was slightly inhibited by 50 mM heliotrine and by 33 mM lasiocarpine.

(c) *Effect of other pyrrolizidine alkaloids on mitochondrial oxidations*

Several other pyrrolizidine alkaloids from different plants were tested to determine whether inhibition of pyridine nucleotide-requiring enzyme systems, and stimulation of succinoxidase were properties common to pyrrolizidine alkaloids. The alkaloids, platyphylline (1) and sarracine (2) from *Senecio spp.*, monocrotaline (3) from *Crotalaria spp.* and supinine (4) from *Tournefortia sarmentosa* and *Heliotropium spp.* differ in structure from lasiocarpine (5) and heliotrine (6) as follows:

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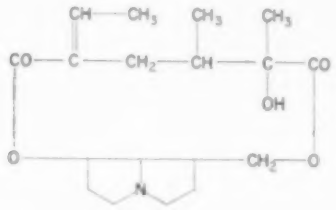
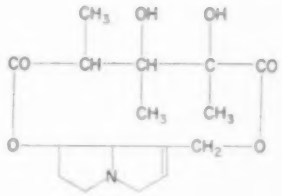
(1) platynecine + senecic acid

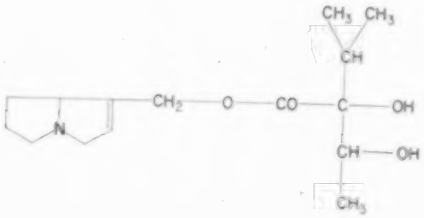


(2) angelic acid + platynecine + sarracenic acid

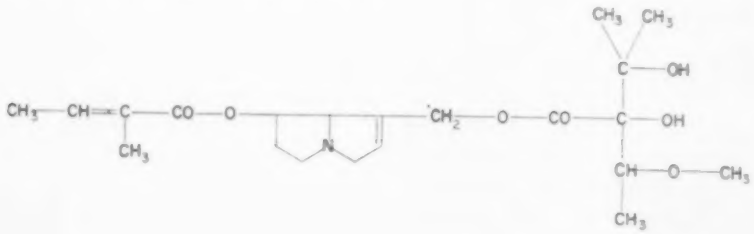
(3) retronecine + monocrotalic acid



(4) supinidine + trachelanthic acid



(5) angelic acid + heliotridine + lasiocarpic acid



(6) heliotridine + heliotric acid

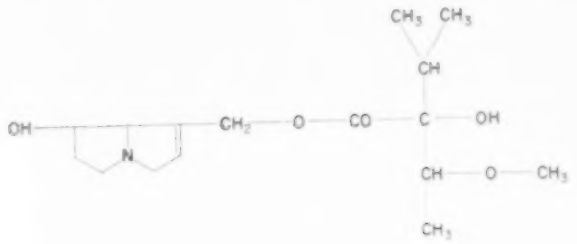


Table 3 shows that a similar effect on mitochondrial metabolism was exerted by each of these alkaloids. Pyridine nucleotide-dependent oxidation of L-malate was inhibited and succinate oxidation was increased or not affected.

(d) *Effect of heliotridine and heliotric acid on* L-*malate oxidation*

The structural components of heliotrine, heliotridine and heliotric acid were investigated to determine which portion of the alkaloid inhibited enzyme systems.

TABLE 3. EFFECT OF OTHER PYRROLIZIDINE ALKALOIDS ON OXIDATIONS BY MITOCHONDRIA

Additions, mM		Oxygen uptake (μl/30 min)	
		L-Malate 10 mM	Succinate 10 mM
None		31	74
Platyphylline	67	3	80
	33	12	84
	6·7	23	—
Supinine	67	1	78
	33	2	72
	6·7	25	76
Monocrotaline	67	4	74
	33	25	90
	6·7	31	—
Sarracine	67	1	—
	6·7	3	58
	3·3	10	118
	0·67	27	90

System: As in Table 1.

Heliotric acid was found to be without effect on L-malate oxidation but heliotridine was inhibitory in the same order as the complete alkaloid (Table 4).

(e) *Effect of cofactors on inhibition of oxidations*

Inhibition of pyridine nucleotide-requiring enzymes but not of the succinoxidase system suggested that loss or inactivation of mitochondrial nucleotides was the mechanism of inhibition. Addition of 0·5 mM DPN in 40 mM nicotinamide significantly reversed the inhibition of oxidation of citrate, L-glutamate, α-oxoglutarate and L-malate by lasiocarpine or heliotrine. Better protection was afforded by the addition of

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TABLE 4. EFFECT OF HELIOTRIDINE AND HELIOTRIC ACID ON L-MALATE OXIDATION

Additions	Oxygen uptake (μl/30 min.)				
	Control	Heliotridine			
		(67 mM)	(50 mM)	(33 mM)	(16·7 mM)
L-Malate (10 mM)	38	4	6	19	35
		Heliotric acid			
		(67 mM)	(50 mM)	(33 mM)	(16·7 mM)
L-Malate (10 mM)	43	41	41	42	43

System: As in Table 1.

0·67 mM GSH to 0·5 mM DPN and 40 mM nicotinamide as shown in Table 5. Similarly, an increase of DPN to 1·5 mM gave greater reversal of inhibition (Table 5).

(f) *Effect of* EDTA

Some reduction of the inhibition by lasiocarpine or heliotrine was obtained by the addition of 1 mM EDTA, although preparations of the alkaloids were free from contamination with calcium or heavy metals. Similarly, the stimulatory effect of the

TABLE 5. EFFECT OF COFACTORS AND EDTA ON INHIBITION OF L-MALATE OXIDATION

Additions	Oxygen uptake (μl/30 min)				
	Control	Cof. 1	Cof. 2	EDTA (1 mM)	EDTA (1 mM) + Cof. 1
None	36	38	33	41	58
Lasiocarpine 6·7 mM	2	23	28	21	50
None	40	54	—	44	50
Heliotrine 33 mM	10	37	—	29	44

System: As in Table 1 + L-malate 10 mM.
Cof. 1 = DPN 0·5 mM, GSH 0·67 mM and nicotinamide 40 mM.
Cof. 2 = DPN 1·5 mM, GSH 0·67 mM and nicotinamide 40 mM.

alkaloids on succinate oxidation was removed by the addition of 1 mM EDTA. Table 5 shows the protective effect of 1 mM EDTA against lasiocarpine or heliotrine inhibition of L-malate oxidation. Greater protection did not result from increasing the EDTA concentration to 2 mM.

Inclusion of 1 mM EDTA, 0·5 mM DPN, 40 mM nicotinamide and 0·67 mM GSH in the reaction mixture almost completely prevented inhibition of L-malate oxidation by either alkaloid (Table 5).

(g) *Effect of pyrrolizidine alkaloids on mitochondrial membrane permeability*

The above data indicated that pyrrolizidine alkaloids inhibited mitochondrial enzyme systems by interfering with electron transport at the pyridine nucleotide level. As the inhibitory effect was reversed by adding excess DPN, the alkaloids appeared to

act either by preventing re-oxidation of reduced pyridine nucleotide or by causing the loss of coenzymes from dependent enzyme systems. In view of the protective action of EDTA against inhibition by the alkaloids and the wellknown stabilizing effect of EDTA on mitochondrial integrity,[13–16] and, in particular its effect on mitochondrial membranes,[17] it seems likely that mitochondrial structure was altered in such a way as to increase membrane permeability with a consequent loss of pyridine nucleotide and other cofactors.

An active measure of mitochondrial permeability may be obtained by following spectrophotometrically at 340 mμ the rate of reduction of DPN which has been added external to intact mitochondria oxidizing a DPN-dependent substrate[18–21] The rate of reduction of DPN is directly proportional to the permeability of mitochondrial membranes to DPN. No effect of lasiocarpine or heliotrine on the rate of DPN reduction coupled to L-malate oxidation could be demonstrated when this system was tested with mitochondria suspended in 0·25 M sucrose or water. These experiments indicated that under the conditions of spectrophotometric as well as manometric experiments the alkaloids do not inhibit L-malate dehydrogenase directly. However, it was realized that the experimental conditions were very different from those obtaining in the system used for respiratory experiments. In particular, the spectrophotometric system contained CN^- to inhibit cytochrome oxidase, semicarbazide to remove oxaloacetate, an excess of added DPN and substrate, a relatively small number of mitochondria, and experiments were carried out at 22° as opposed to 38° for manometric experiments. Mitochondria re-isolated from a respiratory experiment in which they had been subjected to action of 6·7 mM lasiocarpine were found to be more permeable than incubated control mitochondria to the entry of DPN.

An effect of lasiocarpine on mitochondrial permeability was demonstrated in the following manner. Mitochondria equivalent to 800 mg of fresh rat liver were suspended in 5 ml of 0·25 M sucrose containing, where indicated in Fig. 1, 10 mM L-malate plus 10 mM lasiocarpine, and were placed in an incubator at 37° for 30 min. During the incubation the temperature of the reaction mixture rose to 32°. After adding 35 ml of ice-cold 0·25 M sucrose the mitochondria were re-isolated by centrifuging at 34,000 × *g* for 10 min. The mitochondria were then resuspended in 0·25 M sucrose or in water, as required, and permeability was assessed by the spectrophotometric method.[18–20] Fig. 1 shows that incubation of mitochondria in the presence of lasiocarpine increased membrane permeability to the passage of DPN. DPN was reduced much more rapidly by mitochondria which had been pre-treated with lasiocarpine in the presence of L-malate than by mitochondria pre-incubated with L-malate alone. The mitochondria treated with lasiocarpine were apparently even more permeable than those aged by pre-incubation in the absence of a substrate (Fig. 1). Initial differences in optical density shown in the curves (a) and (b) in Fig. 1 were due to reduction of DPN at different rates during the short time between the adding of the enzyme and the taking of the first reading. Lasiocarpine does not absorb light at 340 mμ. The initial optical density at 340 mμ of mitochondria pre-incubated without L-malate was always higher than that of mitochondria pre-incubated with L-malate. Suspension of mitochondria in water instead of in 0·25 M sucrose abolishes selective semipermeability of the membranes. The effect of lasiocarpine was abolished by suspension of mitochondria in water and must therefore have been associated with semipermeability of mitochondrial membranes.

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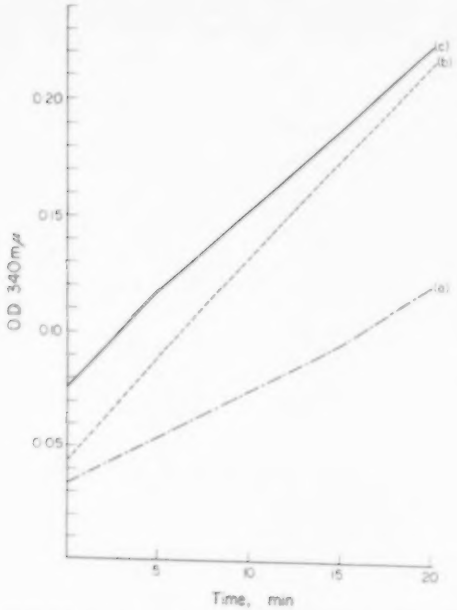

FIG. 1. Mitochondria in 0·25 M sucrose pre-incubated with (a) L-malate 10 mM; (b) L-malate 10 mM + lasiocarpine 10 mM; (c) No addition.
System: NaK phosphate buffer, pH 7·8, 33 mM; KCl 25 mM; $MgSO_4$ 6·7 mM; L-malate 50 mM; semicarbazide HCl (neutralized) 0·17 M; DPN 1·5 mM; KCN 0·2 mM; enzyme, 50 μl 1% mitochondria in 0·25 M sucrose; final volume, 3 ml; gas phase, air; temperature, 22°.

Finally, it was found that mitochondrial permeability increased rapidly under the oxidizing conditions of respiratory experiments. As already described, the alkaloids stimulated succinate oxidation by mitochondria in 0·25 M sucrose. This may be due to the increased permeability of mitochondrial membranes allowing the loss of mitochondrial DPN and thus preventing the production of oxaloacetate which in low concentration inhibits succinic dehydrogenase specifically.[22–24] Lack of any effect of lasiocarpine or heliotrine on succinate oxidation by mitochondria in water provides supporting evidence, as water-disrupted mitochondria would be deficient in DPN and would not be producing oxaloacetate. Further evidence was obtained by adding DPN to mitochondria oxidizing succinate in the presence of 6·7 mM lasiocarpine. DPN added to the suspending fluid enters mitochondria slowly unless membrane permeability is increased.[25] Succinate oxidation by mitochondria in 0·25 M sucrose was slightly inhibited by adding 0·5 mM DPN alone, but was rapidly and greatly inhibited by 0·5 mM DPN plus 6·7 mM lasiocarpine (Table 6). When mitochondria were suspended in water, the addition of DPN alone produced maximum inhibition of succinate oxidation, as shown in Table 6, and lasiocarpine did not further reduce the rate of oxidation when added with or without DPN. Similar results were obtained with heliotrine. The addition of 0·4 mM $CaCl_2$ increased the membrane permeability of mitochondria suspended in 0·25 M sucrose, and produced a pattern of events similar to that resulting from the pyrrolizidine alkaloids. Table 6 shows that Ca^{2+} stimulated succinate oxidation by mitochondria in 0·25 M sucrose and facilitated penetration and

inhibition by DPN. Suspension of mitochondria in water also removed the effect of Ca^{2+} (Table 6). Clearly pyrrolizidine alkaloids, like Ca^{2+}, increased the permeability of mitochondrial membranes to the passage of DPN.[25]

TABLE 6. EFFECT OF LASIOCARPINE, CALCIUM AND DPN ON SUCCINATE OXIDATION

Additions	Oxygen uptake (μl/30 min)			
	Sucrose medium		Water medium	
	No DPN	DPN 0·5 mM	No DPN	DPN 0·5 mM
None	68	62	57	12
Lasiocarpine 6·7 mM	101	47	61	13
$CaCl_2$ 0·4 mM	90	15	68	15
Lasiocarpine 6·7 mM + $CaCl_2$ 0·4 mM	94	19	—	—

System: As in Table 1 + succinate 10 mM. Mitochondria equivalent to 25 mg of fresh rat liver added in 0·2·5 ml of 0·25 M sucrose or water. Results shown are the means of three experiments.

(h) *Effect of lasiocarpine, and heliotrine, on light absorption at 260 mμ by mitochondrial extracts*

Mitochondria, re-isolated after incubation with lasiocarpine or heliotrine and L-malate in the respiratory system shown in Table 1, were extracted at room temperature for 10 min with 5% trichloracetic acid and the optical densities of the extracts were measured at 260 mμ. Pre-treatment with either alkaloid resulted in significant reduction of the acid-extractable material absorbing light at 260 mμ when compared with similar extracts of incubated control mitochondria. If the extracts were made without incubation, the amount of extractable material absorbing at 260 mμ was the same whether alkaloid had been added or not. At low concentrations of the alkaloids, and for periods of up to 50 min, the loss of absorption at 260 mμ was proportional to alkaloid concentration and incubation time. This evidence suggested an abnormal loss of oxidized pyridine nucleotides from the mitochondria during incubation with the alkaloids, and it fitted the mode of action already postulated. Details of the effects of pyrrolizidine alkaloids on mitochondrial pyridine nucleotides will be presented in a subsequent paper.

DISCUSSION

The pyrrolizidine alkaloids, lasiocarpine and heliotrine, from *Heliotropium europaeum*, were found in these studies to inhibit, *in vitro*, oxidative metabolism of liver mitochondria from normal rats. They inhibited specifically those mitochondrial enzyme systems which require pyridine nucleotides for electron transport. Either alkaloid stimulated succinoxidase activity but neither affected cytochrome oxidase activity. Supinine, which also occurs in *Heliotropium europaeum* and several pyrrolizidine alkaloids from other plants exerted a similar effect on mitochondrial metabolism. It seems likely that this is a property common to all pyrrolizidine alkaloids.

The inhibitory locus of the alkaloids appears to be the nitrogen atom of the cyclic nucleus. Oxidation at the ring nitrogen to form the N-oxide completely removes the effects of the alkaloids on mitochondrial oxidations. From a comparison of the

structure of the pyrrolizidine alkaloids having the same effect on mitochondrial metabolism it seems likely that the pyrrolizidine ring is the inhibitory component, as the esterified organic acids differ greatly. Further, heliotridine the cyclic base of heliotrine influences mitochondrial metabolism in the same way as the parent alkaloid, but heliotric acid is without effect.

The inhibition of pyridine nucleotide-requiring enzyme systems, and the protective effect of excess DPN indicated that electron transport is being blocked at the pyridine nucleotide level, either by interference with re-oxidation of pyridine nucleotides or by loss of coenzymes from mitochondrial enzyme systems. The stimulatory effects of the alkaloids on succinate oxidation by mitochondria in 0·25 M sucrose, and the stabilizing influence of EDTA against this effect and against inhibition of nucleotide-dependent enzymes, indicate that the alkaloids may alter mitochondrial structure in a manner which increases membrane permeability, and so permits the loss of cofactors from the mitochondria. It was in fact shown that mitochondrial membrane permeability to the passage of DPN is increased by lasiocarpine or heliotrine under the oxidizing conditions of respiratory experiments. In the experiments, the loss from mitochondria of acid-extractable material absorbing light at 260 mμ. an absorption which is due in part to oxidized pyridine nucleotides, is accelerated by the addition of the alkaloids. Spectrophotometric estimation of DPN reduction coupled to L-malate oxidation, and manometric assay of L-malate and of succinate oxidation, each with water-disrupted mitochondria, failed to show any direct effect of heliotrine or lasiocarpine on L-malic dehydrogenase or on the succinoxidase system. It is thus clear that pyrrolizidine alkaloids can influence mitochondrial metabolism by increasing the permeability of mitochondrial membranes to pyridine nucleotides and, possibiy, other soluble cofactors of respiration. Loss of pyridine nucleotide inactivates dependent enzyme systems, and stimulates succinate oxidation by suppressing the formation of oxaloacetate.[24] The mechanism by which pyrrolizidine alkaloids cause increased mitochondrial permeability, with loss of pyridine nucleotides, will be discussed in a subsequent paper.

The findings of this study support those of Christie[7,11] who observed that inactivation of pyridine nucleotide-dependent oxidations by liver enzymes preceded the appearance of histological evidence of liver necrosis in rats injected with a single large dose of heliotrine. Christie was able to reverse the inhibitions by adding DPN, and he suggested that heliotrine might disorganize tricarboxylic acid cycle activity by increasing mitochondrial permeability and lead to the loss of respiratory cofactors. Thus it is probable that *in vivo* administration of pyrrolizidine alkaloids influence liver mitochondria in the same way as does direct addition of the alkaloid to mitochondrial preparations *in vitro*.

If a dose of pyrrolizidine alkaloid is considered to be distributed evenly throughout the body, the concentration required to produce acute hepatic necrosis is considerably lower (at least ten times) than that required to inhibit mitochondrial oxidations *in vitro*. However, at least 12 hr must elapse after administration before inhibition of liver mitochondrial metabolism is apparent. Accumulation of alkaloid in the liver during this time could easily result in concentrations of the order necessary to inhibit oxidations *in vitro* by liver mitochondria from normal rats. It seems likely that the delayed onset of measurable mitochondrial dysfunction subsequent to a dose of pyrrolizidine alkaloid is in fact due to the time of accumulation of an inhibitory

concentration in the liver. Finally the different chemical and physical conditions *in vivo* may favour the activity of pyrrolizidine alkaloids at lower concentrations than the conditions of *in vitro* experiments.

Greater potency of lasiocarpine than of heliotrine *in vitro* is in agreement with the relative median lethal doses of these alkaloids established by Bull *et al*[5]. However, in the present study, neither heliotrine N-oxide nor lasiocarpine N-oxide was found to affect oxidative metabolism, although the toxicity of lasiocarpine N-oxide to female rats is relatively high.[5] This difference may be due to reduction of the N-oxide to lasiocarpine after *in vivo* administration. Alternatively lasiocarpine N-oxide may itself affect metabolism in the female rat in a yet undetermined manner.

The acute hepatotoxic action of pyrrolizidine alkaloids would seem to result from loss of pyridine nucleotides from the mitochondria, arising from increased permeability. This loss would inactivate enzyme systems which require pyridine nucleotides for electron transfer, and thus disrupt tricarboxylic acid cycle activity.

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THE EFFECTS OF NEUROMUSCULAR BLOCKING AGENTS ON MITOCHONDRIA—I

EFFECTS OF *d*-TUBOCURARINE AND OF COMPLEX IONS ON MITOCHONDRIAL ENZYME SYSTEMS*

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Abstract—*d*-Tubocurarine and some complex ions, e.g. tris-1 : 10-phenanthroline ruthenium-II perchlorate dihydrate, inhibit oxidations by liver mitochondrial enzyme systems which require pyridine nucleotides for electron transfer, and stimulate succinate oxidation by mitochondria in 0·25 M sucrose. The compounds do not affect the activity of cytochrome oxidase or the efficiency of oxidative phosphorylation. Neither *d*-tubocurarine nor 1-Ru (phen)$_3^{++}$ inhibit L-malic dehydrogenase directly, nor does *d*-tubocurarine stimulate succinate oxidation by mitochondria in water. However, 1-Ru (phen)$_3^{++}$ inhibits the succinoxidase system of water-disrupted mitochondria.

d-Tubocurarine and 1-Ru (phen)$_3^{++}$ influence oxidations by mitochondria in 0·25 M sucrose by increasing mitochondrial permeability and accelerating the loss of pyridine nucleotides. Inhibition of L-malate oxidation is largely prevented by adding excess DPN, and slightly prevented by EDTA. Mitochondria take up *d*-tubocurarine during incubation and some, at least, of the alkaloid becomes localized in the insoluble, membraneous fraction.

Active oxidation is necessary both for the development of inhibition by *d*-tubocurarine and for mitochondrial uptake of the alkaloid. The uptake of *d*-tubocurarine and of added DPN by mitochondria oxidizing succinate is prevented by Na amytal. Both increased permeability and *d*-tubocurarine uptake appear to be associated with the re-oxidation of reduced pyridine nucleotides. Similar effects of *d*-tubocurarine, pyrrolizidine alkaloids and complex ions on liver mitochondria and on neuromuscular junctions suggest that the compounds may act similarly at either tissue site and that such action may be referable to the charge carried.

INTRODUCTION

IN AN earlier study,[1] pyrrolizidine alkaloids were found to inhibit impulse transmission across neuromuscular junctions in a manner which resembled in some respects the neuromuscular blocking action of *d*-tubocurarine. Pyrrolizidine alkaloids have been shown also to inhibit the activity of certain enzyme systems of liver mitochondria.[2] The inhibitory locus of the pyrrolizidine alkaloids operative for either neuromuscular or mitochondrial systems appears to be the nitrogen atom of the cyclic base. Oxidation at the ring nitrogen to form N-oxides removes the inhibitory

* Abbreviations used in this paper: AMP, adenosine-5-monophosphate; DPN, diphosphopyridine nucleotide; GSH, reduced glutathione; EDTA, ethylenediaminetetraacetic acid; Ru (phen)$_3^{++}$, tris-1 : 10-phenanthroline ruthenium-II perchlorate dihydrate; Ru (trpy)$_2^{++}$, bis-2 : ′ : 2″-terpyridine ruthenium-II perchlorate hemihydrate.

quality of the pyrrolizidine alkaloids for either system. As *d*-tubocurarine blocks the transmission of impulses at neuromuscular junctions by virtue of its two quaternary nitrogen atoms, it was of interest to determine whether this alkaloid also influenced the activity of mitochondrial enzyme systems.

During the study it became pertinent to investigate the possible effects on mitochondrial enzymes of the chemically stable complex ions,[3] which exert a curare-like effect at neuromuscular junctions by nature of the charge they carry.[4–7] A preliminary account of some of this study has been published.[8]

MATERIALS AND METHODS

Enzyme preparations. Rats were killed by stunning and decapitation. Livers were removed rapidly and immersed in ice-cold 0·25 M sucrose. Homogenates were prepared in 0·25 M sucrose and fractionated by differential centrifuging at 0–1 °.[9] Mitochondria were resuspended in 0·25 M sucrose unless otherwise indicated. Hexokinase was prepared from yeast by a modification[10] of the method of Berger *et al.*[11] The fraction precipitating between 25 per cent and 40 per cent by volume of ethanol was dissolved in 1 per cent glucose solution and stored frozen. The optimal addition for oxidative phosphorylation was determined by assay.

Reagents. Complex ions were obtained as gifts for earlier studies from Dr. Dwyer of the National University, Canberra. AMP, DPN, nicotinamide, GSH, cytochrome *c*, citrate, α-oxoglutarate, L-glutamate, succinate, L-malate, oxaloacetate, octanoate, L-ascorbic acid, EDTA, *d*-tubocurarine, ethanol, sucrose and inorganic reagents were obtained commercially.

Succinic acid was purified by recrystallizing three times from hot water: then dissolved in 1N NaOH and precipitated as sodium succinate with ethanol. Sodium succinate was redissolved and crystallized from 80 per cent ethanol. Octanoic acid was purified by distillation *in vacuo*. L-Ascorbic acid and α-oxoglutaric acid were dissolved and neutralized with NaOH just before use. Other substrates and AMP were neutralized and stored frozen as sodium salts for 3–4 weeks.

Methods. O_2 uptake was measured by Warburg manometers. Spectrophotometric measurements were made with a Beckman model DU instrument. *d*-Tubocurarine was measured by the method of Pride and Smith,[12] and inorganic phosphorus by the method of Fiske and Subba Row.[13]

RESULTS

All experiments were done in duplicate and the results expressed are the means of at least repeated experiments.

(a) *Effect on oxidative metabolism*

(i) *Effect of* d-*tubocurarine on mitochondrial oxidations.* Table 1 shows that 1 mM *d*-tubocurarine inhibited the oxidation by mitochondria of citrate, α-oxoglutarate, L-malate, L-glutamate and octanoate but stimulated the oxidation of succinate. Lower concentrations of *d*-tubocurarine had much less effect on mitochondrial metabolism; 0·5 mM was hardly inhibitory to L-malate oxidation and 0·1 mM not at all so. The inhibitory effect of *d*-tubocurarine was slight during the first 10 min of each experiment but developed progressively to severe inhibition within 30 to 40 min. Experiments in which the alkaloid was added from side-arm compartments of Warburg

flasks during the course of oxdative experiments, demonstrated well the progressive development of inhibition.

TABLE 1. EFFECT OF *d*-TUBOCURARINE ON OXIDATIONS BY LIVER MITOCHONDRIA

Additions	Oxygen uptake (μl/30 min)				
	Control	*d*-Tubocurarine (1 mM)	*l*-Ru (phen)$_3^{2+}$ (0·5 mM)	*d*-Ru (phen)$_3^{2+}$ (0·5 mM)	Ru (trpy)$_2^{2+}$ (0·5 mM)
Citrate 10 mM	95	44	—	—	—
α-Oxoglutarate 10 mM	112	63	—	—	—
Succinate 10 mM	165	222	238	—	—
L-Malate 10 mM	108	55	55	62	73
L-Glutamate 10 mM	108	52	—	—	—
Octanoate 1·67 mM + L-malate 0·67 mM	129	69	—	—	—

System: AMP 1 mM; $MgSO_4$ 6·7 mM; KCl 25 mM; phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; mitochondria equivalent to 100 mg of fresh rat liver were added in 0·5 ml of 0·25 M sucrose except for succinate oxidation where mitochondria equivalent to 50 mg of fresh liver were added in 0·5 ml of 0·25 M sucrose; water to 3·0 ml final volume; 0·1 ml of 20 per cent KOH was placed in the centre well to absorb CO_2; gas phase, air; temperature, 38°; equlibration period 10 min.

d-Tubocurarine was found to affect oxidative metabolism by liver homogenates in 0·25 M sucrose in a similar manner to its effect on isolated mitochondria.

(ii) *Effect of complex ions on mitochondrial oxidations.* The complex ions, *l*- and *d*-Ru (phen)$_3^{2+}$ and Ru (trpy)$_2^{2+}$ inhibited L-malate oxidation by liver mitochondria (Table 1). Also *l*-Ru (phen)$_3^{2+}$, the only complex ion tested for such activity, was found to stimulate succinate oxidation as did *d*-tubocurarine (Table 1). Like *d*-tubocurarine, Ru (phen)$_3^{2+}$ became progressively inhibitory to L-malate oxidation.

(iii) *Effect of* d-*tubocurarine and* l-*Ru (phen)*$_3^{2+}$ *on oxidative phosphorylation.* Neither 1 mM *d*-tubocurarine nor 0·5 mM *l*-Ru (phen)$_3^{2+}$ lowered the efficiency of oxidative phosphorylation by liver mitochondria oxidizing L-malate, although oxidation was reduced by either compound.

(iv) *Effect of* d-*tubocurarine and* l-*Ru (phen)*$_3^{2+}$ *on cytochrome oxidase activity.* The cytochrome oxidase activity of rat liver homogenates in water was assayed by the method of Schneider and Potter[14] in the presence of 1 mM *d*-tubocurarine or of 0·5 mM *l*-Ru (phen)$_3^{2+}$. Neither compound had an effect on cytochrome oxidase.

(v) *Effect of* d-*tubocurarine and* l-*Ru (phen)*$_3^{2+}$ *on* L-*malic dehydrogenase.* The total activity of L-malic dehydrogenase was assayed in aqueous homogenates of rat liver in order to determine whether *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$ had a direct effect on the enzyme. Homogenates were prepared in water and allowed to stand at 0–1° for 45 min to ensure that osmotic rupture of mitochondria had occurred. L-Malic dehydrogenase activity was assayed by the method of Potter.[15] In this method the reaction mixture is supplemented with DPN to substitute for DPN lost from mitochondria due to water-cracking, and with L-glutamate to remove oxaloacetate by transamination to aspartate.

Neither 1 mM *d*-tubocurarine nor 0·5 mM *l*-Ru (phen)$_3^{2+}$ had any effect on L-malic dehydrogenase activity when assayed in this system.

(b) *Reversal of* d-*tubocurarine and of* l-*Ru (phen)*$_3^{2+}$ *inhibition*

(i) *Effect of respiratory cofactors.* Inhibition of mitochondrial enzyme systems requiring pyridine nucleotides for electron transfer and not of the succinoxidase system or of L-malic dehydrogenase directly in the presence of excess DPN indicated that the loss or inactivation of mitochondrial pyridine nucleotides might be involved. Incubation of 0·5 mM DPN, 0·67 mM GSH and 40 mM nicotinamide with the reaction mixture before adding *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$ largely prevented the development of inhibition of L-malate oxidation by either compound. Further, the addition of such a cofactor supplement at the same time as *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$ was also efficient in preventing inhibition of L-malate oxidation as shown in Table 2. The important cofactor in protecting against the inhibitory effects was found to be DPN, which gave good protection alone.

(ii) *Effect of EDTA.* A lesser degree of protection against inhibition of L-malate oxidation by *d*-tubocurarine or by *l*-Ru (phen)$_3^{2+}$ was obtained by the addition of 1 mM EDTA as shown in Table 2. Higher concentrations of EDTA did not give better protection. Inhibition by either compound was still severe when mitochondria were isolated from livers homogenized in 0·25 M sucrose containing 1 mM EDTA and resuspended in either 0·25 M sucrose or 0·25 M sucrose + 1 mM EDTA. The rate of development of inhibition was slightly retarded with mitochondria prepared from sucrose—EDTA homogenates as compared with sucrose homogenates, and further slightly retarded by the inclusion of 1 mM EDTA in the Warburg flasks.

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Table 2. Effect of respiratory cofactors and EDTA on inhibition of L-malate oxidation

Additions	Oxygen uptake (μl/40 min)		
	Control	*d*-Tubocurarine (1 mM)	*l*-Ru (phen)$_3^{2+}$ (0·5 mM)
None	159	42	37
Cofactors	170	141	153
EDTA 1 mM	164	99	76
EDTA 1 mM + cofactors	187	164	159

System: As in Table 1 + L-malate 10 mM. Cofactor supplement: DPN 0·5 mM, GSH 0·67 mM and nicotinamide 40 mM.

The effect of EDTA on *d*-tubocurarine inhibition might possibly be due to altering the Ca : Mg ratio of mitochondria by chelation of Ca^{2+}. However, increased addition of Mg^{2+} had no effect on the rate of development or degree of inhibition of L-malate oxidation by the alkaloid.

Slightly better protection against inhibition by *d*-tubocurarine or by *l*-Ru (phen)$_3^{2+}$ was obtained consistently by the inclusion of EDTA as well as cofactors in the reaction mixture but the difference as compared with cofactors alone was always small (cf. Table 2).

(c) *Mechanism of inhibition by* d-*tubocurarine and by* l-*Ru*(*phen*)$_3^{2+}$

(i) *Effect of substrate.* Mitochondria were incubated for 20 min with 1 mM *d*-tubocurarine in the respiratory system shown in Table 3 without the addition of a substrate. No inhibition of endogenous oxidation was observed, but the rate was very low in any case. L-Malate was then added from side-arm compartments to the reaction mixture. Inhibition of oxidation was not evident for 20–30 min after the addition of L-malate and then developed progressively (as shown in Table 3 under "no substrate"). This showed that the inhibitory effect of *d*-tubocurarine was not due to a direct effect on mitochondria but was dependent upon oxidation by the mitochondria of an added substrate.

Further evidence on the necessity of oxidation of a substrate for *d*-tubocurarine inhibition to be expressed was obtained by studying mitochondria isolated from liver homogenates prepared in 0·25 M sucrose containing 1 mM *d*-tubocurarine. The mitochondria were washed by suspension in 0·25 M sucrose, re-isolated and resuspended in 0·25 M sucrose. Oxidation of L-malate by such mitochondria was normal and the mitochondria were not abnormally susceptible to inhibition by *d*-tubocurarine added to the Warburg flasks.

L-Malate was added to the main compartments of Warburg flasks after pre-incubation of mitochondria with *d*-tubocurarine in the presence of 0·67 mM succinate. Table 3, under "succinate 0·67 mM", shows that severe inhibition of oxidation was apparent at the first 10 min reading after adding L-malate, showing that the substrate oxidized during preincubation need not be that of a DPN-dependent enzyme system. The inhibition was of the same order as that shown after pre-incubation with L-malate (Table 3, under "L-malate 5 mM").

TABLE 3. EFFECT OF SUBSTRATES ON INHIBITION OF L-MALATE OXIDATION

Time (min)	Interval oxygen uptake (μl)					
	No substrate control	*d*-tubocurarine (1 mM)	Succinate (0·67 mM) control	*d*-tubocurarine (1 mM)	L-Malate (5 mM) control	*d*-tubocurarine (1 mM)
10	5	7	35	29	33	30
	Mixing equilibration of 5 min after adding L-*malate from side-arm compartments*					
10	7	10	29	7	27	12
20	11	13	31	12	31	12
30	17	9	23	3	20	1
40	16	7	25	0	19	0

System: As in Table 1. Additions of substrate to the centre compartments are indicated below. L-Malate was added from side-arm compartments 20 min after commencing incubation, including 10 min equilibration, to give a final concentration of 10 mM.

(ii) *Effect on mitochondrial permeability.* Inhibition of pyridine nucleotide-dependent enzyme systems and not of succinoxidase, and protection against inhibition by the addition of DPN, indicated that *d*-tubocurarine and the complex ions led to the loss or inactivation of mitochondrial pyridine nucleotides. Further, the protective effect

of EDTA which is known to stabilize mitochondrial integrity,[18–19] and which appears to act on the mitochondrial membrane,[20] indicated that the inhibitory agents might lead to increased permeability of mitochondrial membranes and thus the loss of pyridine nucleotides.

Mitochondrial permeability may be measured by the rate of reduction of DPN which has been added to mitochondria oxidizing a DPN-dependent substrate.[21–24] The rate of reduction of DPN is regulated by the permeability of mitochondrial membranes to DPN. No effect of 1 mM *d*-tubocurarine or of 0·5 mM *l*-Ru $(phen)_3^{2+}$ could be demonstrated when this system was tested with mitochondria suspended in 0·25 M sucrose or in water. The normal rate of reduction of DPN by water-disrupted mitochondria indicated that under the conditions of these experiments as well as in the manometric assay, neither *d*-tubocurarine nor *l*-Ru $(phen)_3^{2+}$ affected L-malic dehydrogenase activity directly.

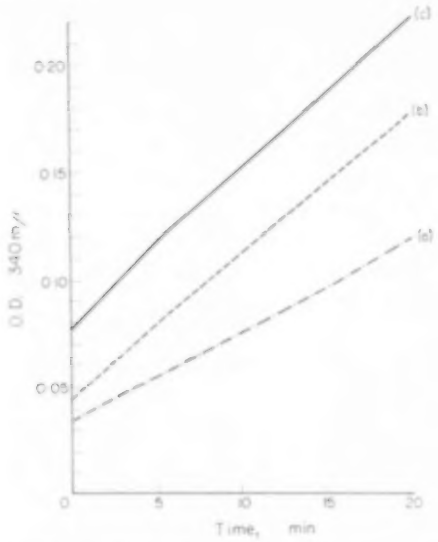

Fig. 1. Reduction of DPN by liver mitochondria in 0·25 M sucrose pre-incubated with: (a) L-malate 10mM; (b) L-malate 10 mM + *d*-tubocurarine 1 mM; (c) no addition.
System: NaK phosphate buffer, pH 7·8, 33 mM; KCl 25 mM; $MgSO_4$ 6·7 mM, L-malate 50 mM; semicarbazide HCl (neutralized) 0·17 M; DPN 1·5 mM; KCN 0·2 mM; enzyme, 50μl 1 per cent mitochondria in 0·25 M sucrose; final volume 3 ml; gas phase air; temperature 22°.

The experimental conditions of such spectrophotometric experiments (cf. Fig. 1) were quite different from those of manometric experiments (cf. Table 1). Furthermore the slow development of inhibition by *d*-tubocurarine or by the complex ions and the dependence upon the oxidation of a substrate indicated that the oxidation–reduction state of the electron transport chain might be a determinant of inhibition. Increased mitochondrial permeability might not be evident except under oxidizing conditions when electrons are moving along the terminal electron transport chain.

Mitochondria re-isolated from manometric experiments in which they were treated with 1 mM *d*-tubocurarine or 0·5 ml *l*-Ru $(phen)_3^{2+}$ were found to be more permeable to DPN than were incubated control mitochondria. This effect was also demonstrated

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in the following way. Mitochondria equivalent to 800 mg of fresh rat liver were suspended in 5 ml of 0·25 M sucrose containing, where indicated in Fig. 1, 10 mM L-malate and 1 mM *d*-tubocurarine, and placed in an incubator at 37 ° for 30 min. During the incubation, the temperature of the reaction mixture rose to 32 °. After adding 35 ml of ice-cold 0·25 M sucrose the mitochondria were re-isolated by centrifuging at 34,000 **g** for 10 min at 0 °. The mitochondria were re-suspended in 0·25 M sucrose or in water, and permeability was assessed by the spectrophotometric method.[21–23] Fig. 1 shows that incubation of mitochondria in the presence of *d*-tubocurarine and L-malate increased permeability to DPN. The rate of reduction of DPN, and thus the increase in permeability, due to *d*-tubocurarine with L-malate is about the same as that resulting from incubation of mitochondria without a substrate (Fig. 1). Initial differences in extinction coefficient shown in the curves for L-malate and L-malate plus *d*-tubocurarine in Fig. 1 would be due to reduction of DPN at different rates during the short time between adding the enzyme and taking the first reading as *d*-tubocurarine does not absorb light appreciably at 340 mμ. Mitochondria pre-incubated without substrate invariably had a higher initial extinction coefficient at 340 mμ than those incubated with substrate. Suspension of the pre-incubated mitochondria in water, which abolishes selective semipermeability of the membranes, instead of 0·25 M sucrose, completely removed any difference of the mitochondria treated with *d*-tubocurarine from controls. The effect of *d*-tubocurarine must therefore have been associated with mitochondrial permeability.

Mitochondrial permeability was found to increase under the conditions of manometric experiments. Both *d*-tubocurarine and *l*-Ru $(phen)_3^{2+}$ were observed to stimulate succinate oxidation by mitochondria in 0·25 M sucrose. This indicated the possibility of increased permeability of mitochondrial membranes allowing the loss of mitochondrial DPN, and blocking of the tricarboxylic acid cycle at L-malate thus preventing the production of oxaloacetate, which in low concentration inhibits succinic dehydrogenase specifically.[25–27] Lack of an effect in most experiments and only slight inhibition in others of *d*-tubocurarine on succinate oxidation by mitochondria in water favoured this explanation, as water-disrupted mitochondria would be deficient in DPN and therefore not producing oxaloacetate. Confirmatory evidence was obtained by adding DPN to mitochondria oxidizing succinate in the presence of 1 mM *d*-tubocurarine. DPN added outside intact mitochondria enters slowly unless permeability is increased.[28] Because of the slow penetration of DPN into mitochondria in 0·25 M sucrose, succinate oxidation was only slightly inhibited by adding 0·5 mM DPN alone (Table 4). However, the addition of 1 mM *d*-tubocurarine as well as 0·5 mM DPN led to severe inhibition of the oxidation of succinate by mitochondria in 0·25 M sucrose (Table 4). Table 4 also shows that when mitochondria were disrupted osmotically by suspension in water, DPN alone produced maximum inhibition of succinate oxidation and *d*-tubocurarine did not significantly alter the rate of oxidation when added with or without DPN. Similar results were obtained with 0·5 mM *l*-Ru $(phen)_3^{2+}$ except that the ion itself was inhibitory to succinate oxidation by water-cracked mitochondria. Ru $(phen)_3^{2+}$ stimulated succinate oxidation by mitochondria in 0·25 M sucrose when added alone and facilitated inhibition by DPN (Table 4).

The addition of 0·4 mM $CaCl_2$ to a system oxidizing succinate by mitochondria produced a pattern of events similar to those resulting from *d*-tubocurarine.[28] Ca^{2+}

stimulated succinate oxidation by mitochondria in 0·25 M sucrose and facilitated the penetration by added DPN and consequent inhibition of oxidation (Table 4). Suspension of mitochondria in water also removed the effect of Ca^{2+}. The inhibition of succinate oxidation by mitochondria in 0·25 M sucrose resulting from Ca^{2+} plus DPN was not significantly increased by adding either 1 mM *d*-tubocurarine or 0·5 mM *l*-Ru $(phen)_3^{2+}$ as shown in Table 4.

Table 4. Effect of *d*-tubocurarine, calcium and DPN on succinate oxidation

Additions	Oxygen uptake (μl/30 min)			
	Sucrose medium		Water medium	
	No DPN	DPN 0·5mM	No DPN	DPN 0·5 mM
None	161	137	132	42
d-Tubocurarine (1 mM)	220	53	112	34
l-Ru $(phen)_3^{2+}$ (0·5 mM)	228	30	22	27
$CaCl_2$ (0·4 mM)	212	37	134	30
d-Tubocurarine (1 mM) + $CaCl_2$ (0·4 mM)	216	35	—	—
l-Ru $(phen)_3^{2+}$ (0·5 mM) + $CaCl_2$ (0·4 mM)	217	31	—	—

System: As in Table 1 + succinate 10 mM. Mitochondria equivalent to 50 mg of fresh rat liver were added in 0·5 ml of 0·25 M sucrose or water.

The stimulatory effect of *d*-tubocurarine on succinate oxidation and inhibition in the presence of DPN was present when mitochondria were isolated from homogenates prepared in 0·88 M sucrose instead of the usual 0·25 M sucrose and resuspended in 0·25 M sucrose. However, addition of sucrose to the Warburg flask to give a final concentration of 0·5 M eliminated both the capacity of the succinoxidase system to be stimulated by *d*-tubocurarine or inhibited by *d*-tubocurarine plus DPN.

Clearly *d*-tubocurarine and *l*-Ru $(phen)_3^{2+}$, like Ca^{2+}, increased the permeability of mitochondrial membranes to DPN.

(d) *Uptake of* d-*tubocurarine by mitochondria*

(i) *Absorption of light at* 260 mμ *by, and* d-*tubocurarine content of, mitochondrial extracts.* In order to determine whether oxidized pyridine nucleotides were lost from mitochondria during incubation with *d*-tubocurarine, mitochondria were recovered from Warburg flasks after respiratory experiments and extracted with 5 per cent TCA as follows:

The contents of Warburg flasks were transferred quantitatively to nylon centrifuge tubes using a Pasteur pipette and washing the flasks out thoroughly with ice-cold, 0·25 M sucrose. The volume in the centrifuge tube was made to 40 ml with ice-cold 0·25 M sucrose, and mitochondria were re-isolated by centrifuging at 34,000 g for 10 min at 0 °. The supernatant was discarded and the inside of the tubes dried with filter paper taking care not to touch the mitochondrial pellet. Mitochondria were triturated with a glass rod in 5 ml of 5 per cent TCA, and allowed to stand at room temperature for 10 min. The contents of the nylon tubes were transferred to glass centrifuge tubes and centrifuged at 2000 rev/min for 10 min in an M.S.E. Multiplex centrifuge. The supernatant was collected, neutralized with 2 N NaOH, and made to

6 ml. Absorption of light by the extracts was read at 260 mμ with a light path of 1 cm against a reagent blank of TCA, NaOH and water.

It was clear from the experiments recorded in Table 5, that incubation of mitochondria in the presence of an oxidizable substrate and *d*-tubocurarine increased the TCA extractable-material which absorbed light at 260 mμ. *d*-Tubocurarine was found to absorb light at 260 mμ and concomitant measurement of the *d*-tubocurarine content of the TCA extracts of mitochondria showed that much at least of the increase in absorption at 260 mμ by such extracts was due to uptake of *d*-tubocurarine by the mitochondria (Table 5). Aliquots of 3 ml were taken from the 5 per cent TCA extracts and developed for the measurement of *d*-tubocurarine at 430 mμ by the method of Pride and Smith.[12] It was realized that this method for *d*-tubocurarine analysis was not highly accurate under the conditions of these experiments, but the method was sufficiently accurate and dependable for the required purpose.

TABLE 5. UPTAKE OF *d*-TUBOCURARINE BY MITOCHONDRIA

Additions	Extinction coefficient				*d*-Tubocurarine uptake (μM/flask)
	Control		*d*-Tubocurarine (1 mM)		
	260 mμ	430 mμ	260 mμ	430 mμ	
None	0·126	0·012	0·129	0·012	0·00
L-malate (10 mM)	0·202	0·011	0·414	0·037	0·34
Succinate (10 mM)	0·148	0·008	0·197	0·023	0·14
Succinate (10 mM) + amytal (1·8 mM)	0·190	0·010	0·116	0·013	0·00
Succinate (10 mM) + DPN (0·5 mM)	—	—	0·298	0·026	0·18
Succinate (10 mM) + DPN (0·5 mM) + amytal (1·8 mM)	—	—	0·137	0·014	0·00

System: As in Table 1. Mitochondria equivalent to 100 mg of fresh liver were added in 0·5 ml of 0·25 M sucrose. Incubation time was 20 min after 10 min equilibration. Mitochondria were re-isolated, extracted with 5 per cent TCA and analysed for *d*-tubocurarine as described in the text.
d-Tubocurarine uptake was calculated from E_{430} readings with correction for E_{430} of mitochondrial extracts in absence of *d*-tubocurarine.

Table 5 shows that mitochondria did not take up *d*-tubocurarine when incubated without added substrate. However, incubation with either L-malate or succinate led to an appreciable uptake of *d*-tubocurarine as measured by extinction coefficients at both 260 mμ and 430 mμ. The uptake of *d*-tubocurarine was consistently greater from incubation with L-malate than with succinate (Table 5). Incubation was found to be necessary for *d*-tubocurarine uptake to occur, as immediate extracts of mitochondria which had been added to reaction mixtures as in Table 5 containing L-malate or succinate and *d*-tubocurarine were not different in absorption either at 260 mμ or at 430 mμ from other mitochondria in the absence of *d*-tubocurarine.

The presence of 1 mM EDTA in the respiratory system did not affect the uptake of *d*-tubocurarine by mitochondria.

Oxidation of L-malate was accompanied by a peak of uptake of *d*-tubocurarine by mitochondria at about 30 min after commencing incubation, including 10 min equilibration. Longer incubation led to the loss of material absorbing light at 260 mμ but

less loss of *d*-tubocurarine as measured at 430 mμ. For example, the experiments comprising group 1 of Table 6 showed that after 50 min incubation with L-malate and *d*-tubocurarine, mitochondrial extracts had an extinction coefficient at 260 mμ of 0·218, as compared with 0·414 after 30 min incubation as shown in Table 5.

d-Tubocurarine uptake by mitochondria during the oxidation of succinate might be related to the reduction and subsequent re-oxidation of pyridine nucleotides, even though the succinoxidase system is not dependent upon pyridine nucleotides for electron transport. Chance and Hollunger[29] have shown that mitochondrial pyridine nucleotides are reduced rapidly during the oxidation of succinate. The uptake of *d*-tubocurarine by mitochondria accompanying succinate oxidation was found to be prevented by adding 1·8 mM Na amytal, which inhibits the re-oxidation of reduced pyridine nucleotides[30] (Table 5). Further it was shown that the increased absorption at 260 mμ resulting from incubation of mitochondria with succinate, DPN and *d*-tubocurarine, and the *d*-tubocurarine uptake as measured at 430 mμ are prevented by the addition of amytal (Table 5).

(ii) *Localization of* d-*tubocurarine in mitochondria*. The coincidence of *d*-tubocurarine uptake with activity of the succinoxidase system, which is associated with mitochondrial membranes,[31] and the effect of *d*-tubocurarine on mitochondrial membrane permeability, indicated that the uptake of *d*-tubocurarine might be by some component of the mitochondrial membrane.

Succinate was oxidized by mitochondria which had been suspended in water and stood for 1 hr at 0°. to ensure water-cracking. The mitochondrial fragments were re-isolated by transferring the Warburg flask contents quantitatively to centrifuge tubes, and centrifuging at 45,000 **g** for 20 min at 0°. The mitochondrial pellet was then analysed for *d*-tubocurarine and for material absorbing at 260 mμ.

Table 6. Localization of *d*-tubocurarine in mitochondria

Group	Additions	Extinction coefficient			
		Control		*d*-Tubocurarine (1 mM)	
		260 mμ	430 mμ	260 mμ	430 mμ
1	L-Malate (10 mM)	0·174	0·010	0·218	0·027
2	L-Malate (10 mM)	0·044	0·011	0·132	0·024
3	Succinate (10 mM)	0·083	0·010	0·116	0·016

System: As in Table 1. Mitochondria equivalent to 100 mg of fresh liver were added in 0·5 ml of 0·25 M sucrose for L-malate oxidation and 0·5 ml of water for succinate oxidation.

Incubation times were 40 min for L-malate oxidation and 30 min for succinate oxidation after 10 min equilibration, respectively. Mitochondria were re-isolated as described in text.

Table 6 shows that very little uptake of *d*-tubocurarine occurred during succinate oxidation by water-cracked mitochondria. However, a slight uptake was recorded in all experiments.

This aspect was further investigated by oxidizing L-malate with mitochondria in 0·25 M sucrose in the presence of *d*-tubocurarine. The contents of the Warburg flasks were washed into centrifuge tubes with ice-cold 0·25 M sucrose or water. Water or

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0·25 M sucrose was added to 40 ml and the centrifuge tubes stood for 90 min at 0–1 ° to allow osmotic disruption of those mitochondria which had been suspended in water. The tubes were centrifuged at 45,000 g for 20 min at 0 ° and the mitochondrial pellet analysed for material absorbing at 260 mμ and for *d*-tubocurarine as described.

Suspension of the mitochondria in water resulted in a great loss of 260 mμ absorbing material (group 2, Table 6), as compared with mitochondria suspended in 0·25 M sucrose (group 1, Table 6), but almost no loss of *d*-tubocurarine. This suggested that *d*-tubocurarine was bound to the membranous portion of mitochondria.

DISCUSSION

The neuromuscular blocking agents, *d*-tubocurarine, and the complex ions, *l*- or *d*-Ru (phen)$_3^{2+}$ and Ru (trpy)$_2^{2+}$, have been shown to affect the metabolism of liver mitochondria in a manner similar to the effect of pyrrolizidine alkaloids,[2] which are also neuromuscular blocking agents.[1] Each of these compounds inhibits the oxidations of substrates by mitochondrial enzyme systems which require pyridine nucleotides for electron transfer, and stimulates the oxidation of succinate by mitochondria in 0·25 M sucrose.

Inhibition of oxidations by *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$, the complex ion which was chosen for detailed study, is prevented by the addition of DPN to the reaction mixture of Warburg flasks. Some protection against inhibition is afforded also by EDTA. In both respects, the effects of *d*-tubocurarine and *l*-Ru (phen)$_3^{2+}$ resemble that of the pyrrolizidine alkaloids.

Neither *d*-tubocurarine nor *l*-Ru (phen)$_3^{2+}$ had a direct effect on the activity of L-malic dehydrogenase under the conditions of either manometric or spectrophotometric experiments. *d*-Tubocurarine also was without effect on the succinoxidase system when the permeability of mitochondria was increased by suspension in water instead of in 0·25 M sucrose. In these respects, too, the compounds resemble pyrrolizidine alkaloids. However, unlike *d*-tubocurarine or the pyrrolizidine alkaloids, *l*-Ru (phen)$_3^{2+}$ inhibits succinoxidase activity of mitochondria suspended in water.

The effects of *d*-tubocurarine and of *l*-Ru (phen)$_3^{2+}$, like the effects of pyrrolizidine alkaloids, on oxidative metabolism of liver mitochondria suspended in 0·25 M sucrose are probably due to the agents increasing the permeability of mitochondrial membranes, and thus accelerating the loss of mitochondrial pyridine nucleotides. Loss of pyridine nucleotides from mitochondria inactivates those enzyme systems which are dependent upon pyridine nucleotides for electron transfer and stimulates succinoxidase activity by blocking the formation of oxaloacetate, which inhibits succinic dehydrogenase, at the DPN-requiring step catalysed by L-malic dehydrogenase.[27]

For *d*-tubocurarine and *l*-Ru (phen)$_3^{2+}$ to increase mitochondrial permeability and inhibit pyridine nucleotide-dependent enzyme systems, the mitochondria need to be oxidizing an added substrate. The substrate need not be one oxidized by enzymes which require pyridine nucleotides. The dependence upon the presence of a substrate for the development of inhibition, indicated that the effects of *d*-tubocurarine and *l*-Ru (phen)$_3^{2+}$ might be linked to the state of oxidation and reduction of components of the terminal electron transport chain. As neither agent inhibits cytochrome oxidase or the succinoxidase system, the most likely point at which they might act appeared to be the pyridine nucleotide level. The ability of succinate oxidation to predispose mitochondria to inhibition of L-malate oxidation is not at variance with this

possibility, as it is known[29] that mitochondrial pyridine nucleotides are rapidly reduced when succinate is oxidized, although the succinoxidase system does not require the coenzymes for electron transport.

Mitochondria incubated with *d*-tubocurarine have been found to take up appreciable quantities of the alkaloid. A portion, at least, of the *d*-tubocurarine appears to become localized in the insoluble, membranous fractions of mitochondria. The uptake requires the oxidation of an added substrate and is greater for the DPN-dependent oxidation of L-malate than for succinate oxidation. However, *d*-tubocurarine uptake does occur when succinate is oxidized, and the addition of amytal prevents the uptake, although succinate oxidation is not altered. Amytal inhibits the re-oxidation of reduced DPN[30]. The uptake of *d*-tubocurarine by mitochondria appears then to be associated with the re-oxidation of reduced pyridine nucleotide.

It is possible that the reactions reponsible for the uptake of *d*-tubocurarine are also concerned in increasing mitochondrial permeability and causing the loss of pyridine nucleotides from dependent enzyme systems. Further evidence on this point is supplied by the experiments in which *d*-tubocurarine was incubated with mitochondria which were oxidizing succinate in the presence of added DPN. TCA extracts of such mitochondria absorbed more light at 260 mμ than could be accounted for by the uptake of *d*-tubocurarine and so was probably due to the uptake of added DPN by the mitochondria. Both the uptake of added DPN and of *d*-tubocurarine were prevented by adding amytal to this system, showing that the re-oxidation of reduced DPN was in some manner involved in producing both increased mitochondrial permeability to DPN and to the uptake of *d*-tubocurarine.

Following an initial increase due to uptake of *d*-tubocurarine, the concentration of material absorbing light at 260 mμ in TCA extracts of mitochondria falls progressively with the period of incubation. The loss of 260 mμ absorbing material is not accounted for by the loss of *d*-tubocurarine and would seem likely to be due to the loss of oxidized pyridine nucleotides as was found to occur with the pyrrolizidine alkaloids.[2] A study of the effects of *d*-tubocurarine, *l*-Ru (phen)$_3^{2+}$ and the pyrrolizidine alkaloids on mitochondrial nucleotides will be presented in a subsequent paper.

In the case of pyrrolizidine alkaloids, it was found that the nitrogen atom of the cyclic base was involved in producing inhibitory effects both on liver mitochondrial metabolism[2] and on the transmission of impulses across neuromuscular junctions.[1] *d*-Tubocurarine is a neuromuscular blocking agent by nature of its two quaternary nitrogen atoms and has now been shown to affect liver mitochondrial oxidations in the same manner as do the pyrrolizidine alkaloids.[1] The complex ions are neuromuscular blocking agents due to the charge which they carry[3-7] and have also been found to influence mitochondrial metabolism in a manner similar to the pyrrolizidine alkaloids and to *d*-tubocurarine. Under appropriate oxidizing conditions, the three groups of agents increase mitochondrial permeability to the movement of pyridine nucleotides. The question of whether this is due to a general increase in membrane permeability or a specific increase in permeability to pyridine nucleotides is being investigated. The effect of the agents at neuromuscular junctions is also thought to be at the membrane level. Lower effective concentrations at the neuromuscular junction than with liver mitochondria may be due to greater accessibility or susceptibility to the drugs or to unsuitability of the *in vitro* conditions for optimal demonstration of the effects on liver mitochondria.

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d-Tubocurarine, pyrrolizidine alkaloids and the complex ions possibly produce their effects by the same mechanism on liver mitochondria and on neuromuscular junctions. This mechanism may be related to the charge carried by the nitrogen atoms of the alkaloids in the same way as the charge on the complex ions is effective and may be associated with derangement of membrane functions by such charge.

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SHORT COMMUNICATIONS

On the Mg^{2+} – dependency of the mitochondrial ATPase activation by two azocarcinogens and a related cytotoxic styrylquinoline*

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FRESHLY-ISOLATED liver mitochondria possess a latent ATP-splitting activity which can be manifested by compounds such as DNP or by conditions which interfere with the structural intactness of the particles (e.g. ageing). The first type of ATPase, the DNP-activated ATPase, is independent of Mg^{2+}, whereas the second one, operative in structurally disorganized particles, is largely dependent on Mg^{2+} for its activity.[1] Since the mitochondrial ATPase assay is usually carried out in a medium containing Mg^{2+}, no distinction between the two possible kinds of effect is made. However, once a biologically-active compound has been found to induce ATPase activity in isolated mitochondria, it may be of interest for the study of its mode of action to know whether the compound acts by a DNP-like type of effect or, as may be inferred from the existence of the second type of ATPase, by interference with some component governing the structural organization of the particles.

Previous investigations in this laboratory on styrylquinolines and related compounds[2] and on azobenzene derivatives[3] have shown that some of the members of these series, which are biologically active either as cytotoxics or as hepatic carcinogens, are effective ATPase activators in mouse and rat liver mitochondria. The present note describes the difference in the mode of action, as formulated above, by which the anti-tumour drug DSQ and the hepatic carcinogen DAQ bring about the latter

TABLE 1. MITOCHONDRIAL ATPASE ACTIVATION BY DNP, DSQ, DAQ AND AAT IN THE PRESENCE AND ABSENCE OF Mg^{2+}

Addition	Phosphorus released (μg)					
	In the presence of Mg^{2+} after			In the absence of Mg^{2+} after		
	5 min	10 min	20 min	5 min	10 min.	20 min
None	0	0	7	0	0	0
DNP	39	62	113	49	79	129
DSQ	25	49	90	4	12	26
DAQ	30	52	93	35	58	98
AAT	31	61	105	0	3	7

Conditions described previously[2] (in Fig. 1 of this reference P_i should be read as phosphorus).

DNP: 10^{-4} M, all other compounds: $1{\cdot}3 \times 10^{-4}$ M.

effect (Table 1). ATPase stimulation in rat liver mitochondria by DSQ has been found to be largely dependent on the presence of Mg^{2+}, whereas that by DAQ was not. The molecular structure of the two compounds is similar except that in DSQ an ethylene and in DAQ an azo bridge link the benzene and quinoline rings. However, the presence of an azo bridge *per se* does not necessarily decide whether the ATPase stimulation by a given compound is independent of Mg^{2+} since the hepatic carcinogen AAT activated the ATPase only in the presence of Mg^{2+} (Table 1). The latter result also shows that different hepatic carcinogens, which activate the mitochondrial ATPase, may do so through a different

* Abbreviations used: ATP = adenosine triphosphate; DNP = 2 : 4-dinitrophenol; DSQ = 4-(4′-dimethylaminostyryl)quinoline; DAQ = 4-(4′-dimethylaminophenyl)azoquinoline; AAT = *o*-aminoazotoluene.

mechanism. The present results may thus allow the conclusion that DAQ exhibits a DPN-like type of effect on the mitochondrial ATPase, whereas DSQ and AAT appear to interact primarily with some component or reaction governing the structural integrity of the particles.

In view of the reverse relationship between water uptake by and structural integrity of isolated mitochondria, it is of interest that DNP has been shown[4, 5] to counteract the spontaneous and thyroxine-induced swelling of liver mitochondria incubated in the absence of oxidizable substrate and cofactors, as measured by (changes in) the optical density at 520 mμ of such suspensions. In similar experiments carried out with DAQ and DSQ a small increase, respectively a decrease, of the $o.d._{520}$ of the mitochondria, as compared with that of the controls, was observed. However, an interpretation of the results in terms of mitochondrial shrinkage or swelling was difficult, if not impossible, in view of the limited solubility of the compounds (approximately one-third of the added $1{\cdot}5 \times 10^{-4}$ was in real solution); part of the finely-dispersed compound may become attached to the particles and thus change the optical density of the suspension (measured at 660 mμ in the present case in view of the absorbency of DAQ at 520 mμ). However, in the case of AAT, which was in complete solution, a marked swelling (decrease of 30–50 per cent in $o.d._{520}$) has been noted previously.[3] It has also been reported[3] that DSQ causes a notable swelling of liver mitochondria when Mg^{2+} was present. This effect, i.e. fall in optical density which was interpreted as swelling, has now been found by phase contrast microscopical examination to be due to an agglutination of the particles. Addition of Mg^{2+} (as chloride, 5×10^{-3} M) to the control suspensions resulted in many very small aggregates of mitochondrial bodies without causing a change in the optical density of the suspension as compared with the controls. Addition of DSQ and Mg^{2+} gave rise to the formation of elaborate aggregates of particles and a marked fall in the optical density (60 per cent decrease). In the presence of DAQ and Mg^{2+}, however, no change in the optical density of the mitochondrial suspension was observed, the agglutination of the particles being less than in the former case but still more extensive than in the controls receiving Mg^{2+} only. Since DSQ or DAQ did not cause an agglutination of the mitochondria in the absence of Mg^{2+} these compounds, and DSQ especially so, appear to accelerate the agglutination of mitochondria incubated in the presence of Mg^{2+}.

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Variations in toxicity of some halogen derivatives of acetic acid in rats

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Some halogen derivatives of acetic acid have been used extensively in biological systems and show a degree of toxicity. For example monoiodoacetate acts as an alkylating agent for sulphydryl groups[1] and monofluoroacetate is toxic because it is a precursor of fluorocitrate, which in turn is an inhibitor of aconitase.[2] Because of the lipotropic properties of ethyl trichloroacetate[3, 4] other halogen derivatives of acetate were examined for this property, and in this study their relative toxicity in young rats has been noted.

Young rats (60–80 g) of both sexes were kept on a choline deficient diet which provided 8 per cent casein and 12 per cent gelatin in the food.[4] From the commencement of the experiment (unless otherwise stated) the rats received subcutaneous injections of the ethyl ester of one of the di- or tri-halogen derivatives of acetic acid. The ethyl esters were used rather than the neutralized acids, since the esters should not be excreted as quickly as the salts and hence would provide a higher level of halogen compound in the animal for a longer time. In addition the use of the esters prevented the difficulties involved in injecting large volumes of water soluble salts.

Some typical results are summarized in Table 1, which shows that rats tolerated relatively large doses of di- or tri-halogen esters. In the doses given both the di- or tri-fluoroacetate esters were

TABLE 1. TOXICITY OF SOME HALOGEN DERIVATIVES OF ACETIC ACID IN RATS

Derivatives	No. Rats	Sex	Dosage (mM/100 g)	Total dosage over injection period (mM)	Deaths within injection period
ethyl trifluoroacetate	10	M + F	0·72	10·9	0
	10	M + F	0·72	14·5	0
ethyl trichloroacetate	13	F	1·45	29	2 after 4 injections
	10	M	0·72	10·9	0
ethyl tribromoacetate	10	M	0·52	4·7	1 after 3 injections
ethyl difluoroacetate	10	M	0·66	13·2	1 after 15 injections
ethyl dichloroacetate	5	F	1·44	8·6	2 after 2 injections 2 after 4 injections
	5	M + F	1·44*	1·4	5 after 1 injection
ethyl dichloroacetate†	5	F	1·44	8·6	0
	8	F	1·44	7·2	0

* Rats were in established choline deficiency.
† Rats fed a commercial rat cube which was used for breeding colony.

non-lethal, and were in marked contrast to the lethal oral dose of 0·06 mM/kg for monofluoroacetate reported by Peters.[2] The trichloroacetate was tolerated without signs of distress at doses which were in agreement with the low oral toxicity found by Woodard, Lange, Nelson and Calvery.[5] These authors found that the oral LD_{50} dose for monochloroacetate was 0·08 mM/kg about 1/40 of the lethal dose of trichloroacetate.

The toxicity of the dichloroacetate ester was interesting, for on the experimental dietary regime, with or without added choline, the ester was lethal at the dose given. When the same amount of ethyl dichloroacetate was given to rats of the same size fed the usual diet given to the breeding colony, the ester was not lethal. The difference between the two diets was particularly marked in the quality of protein provided; the breeding diet provided protein which supported rapid growth and reproduction of rats. The toxicity appeared to be depressed when an adequate level of essential amino acids was supplied in the diet. Whether protein was needed to form a detoxication complex with the acid, or for replacement of damaged tissue, has not been investigated. An alternative possibility is that liver halidase[6] may remove one chlorine atom to form monochloroacetate which would combine with tissue-SH groups.

The fluoro derivatives produced more necrotic tissue at the site of injection than was produced in animals given the chloro derivatives. This damage might result from the local accumulation of free acid which could result from the hydrolysis of the ester. Gorin, Pierce and McBee[7] have shown that the ethyl esters of both di- and tri-fluoroacetic acid break down rapidly even in an initially neutral aqueous medium. On the other hand ethyl dichloroacetate has been shown to be relatively difficult

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to hydrolyse,[5] so that local concentrations of free acid may not build up with the chloroacids. However, despite this necrotic damage, the ester-treated animals with the exception of those given ethyl tribromoacetate, increased in body weight during the period of injections at rates which were only slightly less than those of uninjected controls.

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Dimethanesulphonic Acid Esters of Sugar Alcohols

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THE finding of an interesting degree of tumour inhibiting activity in 1,6-dimethanesulphonyl-D-mannitol (I)[1] and the possibility that this activity might depend upon stereochemical and other structural factors led us to investigate some appropriate isomers and analogues in the sugar alcohol series. In brief, all the following compounds were found to be inactive or very feebly active as inhibitors of growth of the Walker tumour, in comparison with (I).

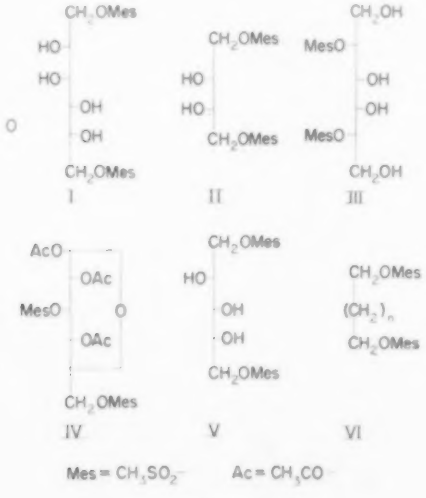

FIG. 1

1,4-Dimethanesulphonyl-erythritol (II); 2,5-dimethanesulphonyl-galactitol (III); 1,2,4-triacetyl-3,6-dimethanesulphonyl-D-glucose (IV); 2,3,4-triacetyl-1,5-dimethanesulphonyl-ribitol; 1,5-dimethanesulphonyl-D-arabitol (V) and its triacetyl derivative; the 2,3,4,5-tetra-acetyl-1,6-dimethanesulphonyl derivatives of D-glucitol and galactitol, which are stereo-isomers of the tetraacetyl derivative of (I), and finally, the enantimorph (or L-form) of (I). 1,4-Dimethanesulphonyl-2,3-diacetyl-DL-threitol, a stereoisomer of (II), has also been made. Since the tetra-acetyl derivative of (I) has approximately the same activity as the parent substance (I) and since both (V) and its triacetyl derivative are very feebly active we have provisionally argued that the 1,6-dimethanesulphonyl derivatives of D-glucitol and galactitol would have little activity, like their tetra-acetyl derivatives.

Comparison of our compounds with the previous series of $\alpha\ \omega$-dimethanesulphonoxyalkanes (VI)[2, 3] shows that in the latter case the biological activity decreases as the chain length increases beyond five carbon atoms (VI; n = 3) although the alkylating ability remains about the same; on the other hand in the sugar alcohol series it appears, upon the basis of the compounds so far examined, that activity increases greatly when the chain length is extended to six carbon atoms although the alkylating ability would remain about the same. Clearly therefore the structural features which determine activity are different in this series and appear to depend very much on the stereochemical structure. Detailed accounts of these investigations will be published later.

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INHIBITION OF DIAMINE OXIDASE *IN VIVO* BY HYDRAZINE DERIVATIVES

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Abstract—The *in vivo* activity of diamine oxidase (DAO) in rat intestine was measured manometrically after intraperitoneal administration of several hydrazine derivatives. The following results were obtained:

1. Iproniazid (72 μM/kg) caused strong inhibition of the enzyme, normal activity being restituted after about 48 hr. Monoamine oxidase (MAO) in brain, however, was inhibited for more than 7 days under the same conditions.
2. DAO inhibition induced by iproniazid could be reversed only partially and after prolonged dialysis.
3. Iproniazid inhibited DAO nearly ten times more strongly than isoniazid, almost equally to semicarbazide but much less than aminoguanidine.
4. There exists no parallelism between the *in vivo* and the *in vitro* potency of the DAO inhibitors investigated.

SEVERAL hydrazine derivatives, e.g. N^2-*iso*propyl*iso*nicotinic acid hydrazide (iproniazid, Marsilid*, are potent inhibitors of monoamine oxidase (MAO) *in vitro*[1–4] and *in vivo*.[4–10] Furthermore these compounds are inhibitors of diamine oxidase (DAO) *in vitro*[1, 11–13] and some of them also *in vivo* (e.g. aminoguanidine, *iso*nicotinic acid hydrazide (isoniazid, Rimifon*)).[14] Iproniazid possibly causes DAO inhibition *in vivo*, too. Evidence for this effect is, however, only indirect.[15–19] Besides, nearly all the results have been obtained by using histamine, the bulk of which is probably not metabolized through DAO in all tissues.[20] Other authors could not find DAO inhibition by iproniazid *in vivo*.[21]

It is conceivable that some of the pharmacological and clinical effects of iproniazid and related compounds in the central nervous system are caused by MAO inhibition.[4,22] Other actions of these drugs (e.g. hypotension, effect on gastric secretion) are, however, difficult to explain by this mechanism and might possibly be due in part to DAO inhibition. In order to elucidate further the mechanism of the clinical and pharmacological action of hydrazine derivatives more information on the *in vivo* activity of these compounds is therefore desirable. In the present paper the effect of iproniazid and some other hydrazine derivatives was studied *in vivo* on intestinal DAO and compared to their action *in vitro*. Thereby cadaverine served as substrate. Furthermore the action of iproniazid on DAO of the small intestine was compared to that on brain MAO.

* Trade name.

METHODS

Experiments in vivo

1. *DAO activity*. After 24 hr fasting, female Wistar rats weighing from 60 to 80 g received a single i.p. injection of the different compounds* in aqueous solution. The inhibitors were applied in three doses, each dose was given to six animals 4 hr prior to decapitation (if not otherwise stated). Untreated rats served as controls. The whole intestines were freed from the contents by perfusion with 25 ml sodium potassium phosphate buffer (Merck p.a. 0·067 M, pH 7·3), softly pressed out and blotted between filter paper. The pooled intestines of each group were cut into small pieces and homogenized in one volume (1 g organ + 1 ml. buffer) of ice-cold buffer by a Servall Omni-mixer (16,000 rev/min for 45 sec). The homogenate was centrifuged at 29,000 × *g* (Servall SS-1) in a cold room for 30 min, the supernatant decanted and kept overnight at + 4 °C after addition of one drop of octyl alcohol (in order to prevent putrefaction and to inhibit MAO).[23]

The activity of the enzyme was measured in Warburg manometers (37·5 °C; O_2 atmosphere, shaking frequency 120/min) during 90 min after addition of the substrate (cadaverine hydrochloride (Roche), final concentration 0·01 M). Each vessel contained enzyme quantities from about 0·8 g fresh tissue, the centre well 10% KOH with filter paper. The activity was expressed as Q_{O_2}, the initial velocity of oxygen uptake calculated as microlitres per hour per gramme wet tissue and corrected for a blank oxygen uptake. Calculation of the ED_{50} (dose for 50 per cent reduction of Q_{O_2}) by regression analysis[24, 25] from at least three series of experiments. The fiducial limits[26] are given for 95 per cent probability.†

2. *MAO activity*. Pretreatment of rats as described under (1). The brain and the medulla oblongata of eight animals, for each dose of the drug, were washed in ice-cold 0·25 M sucrose (Baker p.a.), blotted on filter paper, pooled and homogenized in 4 vols. of 0·25 M sucrose with a Potter–Elvehjem homogenizer (rotating part in Teflon, clearance 0·004–0·006 in. (Kontes Glass Co., Vineland, N.J.)). After a first slow centrifugation (30 min at about 500 × *g*) the supernatant was again centrifuged (30 min at 30,000 × *g*) and the particulate fraction including the fluffy layer resuspended in buffer by stirring with a glass rod. After further centrifugation (20 min at 12,000 × *g*) the "washed" sediment was strongly shaken in buffer until the suspension appeared perfectly homogeneous. All operations were performed at from + 2 ° to 4 °C.

The MAO activity was measured in Warburg manometers as described under (1). The flasks contained particulate matter corresponding to about 0·8 g fresh brain and tyramine hydrochloride (Roche) as substrate (0·0091 M final concentration). The results were expressed as microlitres of O_2 per hour per gramme of original wet tissue, corrected for a blank oxygen uptake. The reaction was linear for at least 60 min.

Experiments in vitro

The enzyme sources were fresh recentrifuged solutions in buffer of DAO acetone

* Iproniazid phosphate and isoniazid, F. Hoffmann-La Roche & Co. Ltd., Basel; semicarbazide hydrochloride, Merck & Co.; aminoguanidine hydrogen carbonate, Fluka A.G., St. Gallen. N^1 : N^2-di*iso*propylhydrazine hydrochloride, *iso*propylhydrazine hydrochloride and N^1-*iso*nicotinyl-N^1 : N^2 di*iso*propylhydrazine as well as N^1 : N^2-di*iso*propylsemicarbazide were synthesized in the Chemical Research Departments of F. Hoffman-La Roche & Co. Ltd., Basel and Nutley, by Drs. O. Straub, P. Zeller and H. H. Fox.

† Calculation and statistical analysis carried out by Mr. G. Güetli.

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powder[27] from hog kidney cortex as well as DAO from rat intestine as described above. The activity of DAO was measured by manometry as described above; the inhibitors were added to the enzyme solution 15 min before addition of the substrate.[28, 29] The efficiency of an inhibitor was expressed by the molar concentration that produced 50 per cent reduction of Q_{O_2} as well as by p_I50 (negative logarithm of that inhibitor concentration). These values were established graphically after measuring the effect of different inhibitor concentrations.

RESULTS

1. *Inhibition of DAO and MAO* in vivo *by iproniazid* (*Fig.* 1)

A single i.p. dose of 72 μM/kg (20 mg/kg) iproniazid phosphate produced a strong inhibition of DAO with a maximal effect between 1½ to 16 hr after administration of

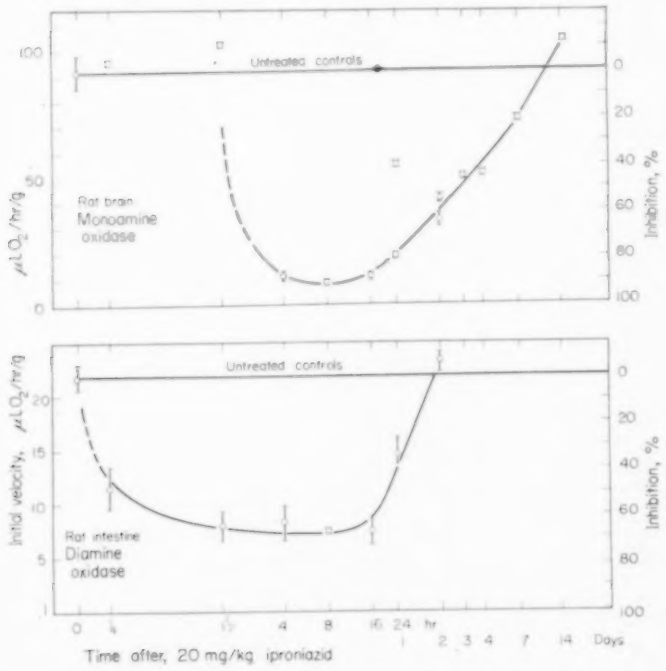

FIG. 1. Iproniazid induced inhibition of MAO and DAO *in vivo* in rats as function of time

Abscissa: time in hours or days in a logarithmic system. Ordinate: left, absolute activity μlO_2 uptake hr^{-1} g^{-1}; right, percentage inhibition. Vertical lines: standard errors of mean values. At zero time 72 μM/kg iproniazid was injected i.p. Each value is derived from a pool of eight animals; the average value of untreated rats killed at the same time intervals served as control. MAO and DAO determinations were carried out in the same animals.

the drug. About 50 per cent inhibition was present already after 15 min, complete recovery of the enzyme activity after about 48 hr. Inhibition of brain MAO in the same animals developed only after 4 hr and was maximal up to 16 hr. Contrary to the

inhibition of DAO the brain MAO was depressed for more than 7 days. The maximum inhibition of brain MAO was of the same order as that of DAO.

2. *Reversibility of DAO inhibition induced by iproniazid* (*Table* 1)

Dialysis of homogenates for 14 hr resulted in a very slight decrease of the DAO activity (14 per cent). Only a very prolonged moving dialysis (5 ml enzyme solution

TABLE 1. INHIBITION OF DAO BY IPRONIAZID *in vitro* WITH AND WITHOUT DIALYSIS

Enzyme preparation		Homogenate of rat intestine	Acetone powder of hog kidney cortex	
Duration of the experiment (hr)		14	14	72
Inhibition (%)	without dialysis	89	98	91
	with dialysis	75	86	49
Reversibility of the iproniazid inhibition (%)		14	12	42

Iproniazid (final concentration 0·01 M) or distilled water (control) were added to a total volume of 5 ml enzyme solution. 10 min later dialysis was started in slowly-moving cellulose casings (Visking Co., Chicago, Ill.) for 14 hr against 2 l. buffer or for 3 days against 5 × 2 l. buffer. All operations were done at from + 2 to + 4 °C. The activity of the samples with iproniazid was compared to controls treated correspondingly.

against 5 × 2 l. buffer within 3 days) decreased the inhibition to a marked degree (42 per cent).

3. *Comparison of DAO inhibitors* in vivo *and* in vitro (*Table* 2)

In vitro the order of potency of the inhibitors was the following: aminoguanidine > *iso*propylhydrazine = semicarbazide ⩾ di*iso*propylhydrazine = isoniazid = iproniazid. Nicotinyldi*iso*proplhydrazine and di*iso*propylsemicarbazide were inactive. DAO from rat intestine as well as purified DAO from hog kidney were equally inhibited by semicarbazide and isoniazid. The affinity of both enzyme preparations for putrescine and cadaverine was also similar. Therefore the *in vitro* results obtained with hog kidney DAO seem to be equivalent to those with enzyme from rat intestine.

In vivo the order of potency of DAO inhibition was quite different from that found *in vitro*, namely: aminoguanidine ⩾ *iso*propylhydrazine > di*iso*propylhydrazine ⩾ iproniazid = semicarbazide > di*iso*propyl-semicarbazide = *iso*nicotinyldi*iso*propyl-hydrazine > isoniazid. Thus, di*iso*propylhydrazine and iproniazid had relatively more potency *in vivo* than *in vitro*, semicarbazide, however, less. The two compounds which showed no effect *in vitro* (*iso*nicotinyldi*iso*propylhydrazine and di*iso*propyl-semicarbazide) had a measurable inhibitory action *in vivo*, even more than isoniazid which was the weakest of all the compounds investigated.

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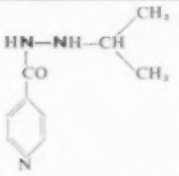
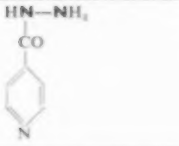
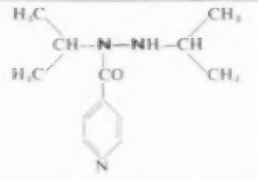
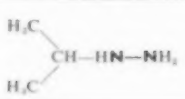
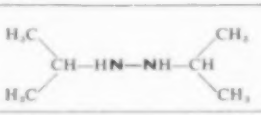
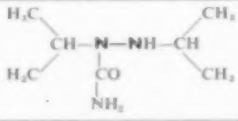
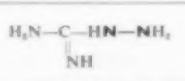
TABLE 2. COMPARISON OF DAO INHIBITORS *in vivo* AND *in vitro*

Method		*in vivo*		*in vitro**		
Inhibitor	Structure	ED_{10} (μM/kg) (fiducial limits)	ED_{50} iproniazid / ED_{50} inhibitor	pI_{50}	Final inhibitor conc. for 50% inhibition (μM)	μM iproniazid / μM inhibitor
Iproniazid		5·5 (2·8–11·6)	1·00	4·2	0·07	1·00
Isoniazid		51·7 (19·0–91·7)	0·11	4·0	0·09	0·8
N^1-*iso*Nicotinyl-N^1: N^2-di*iso*propylhydrazine		19·4 (10·0–66·0)	0·28	<2	>10	<0·01
*iso*Propylhydrazine		1·0 (0·4–1·3)	5·5	6·1	0·00080	87·5
N^1 : N^2-Di*iso*propylhydrazine		2·4 (2·0–3·1)	2·3	4·6	0·025	2·8
N^1 : N^2-Di*iso*propylsemicarbazide		15·2 (8·8–24·5)	0·36	<2	>10	<0·01
Semicarbazide		6·5 (3·5–10·4)	0·85	6·1	0·00085	82
Aminoguanidine		0·07 (0·027–0·12)	78·6	7·2	0·00007	1080

* The values for 50 per cent inhibition described above correspond approximately to the following figures in the literature found under various conditions: iproniazid $pI_{50} \leq 4{\cdot}0$,[1,21] 0·005 M;[18] isoniazid $pI_{50} = 4{\cdot}0$;[1,21] N^1 : N^2-di*iso*propylhydrazine $pI_{50} = 3{\cdot}7$;[20] semicarbazide 0·00004 M,[20] 0·00005 M,[22] 0·00001 M;[18] aminoguanidine 0·00000005 M,[22] 0·0000005 M.[18]

DISCUSSION

The present investigations demonstrate by direct determination of DAO activity in the rat intestine that certain hydrazine derivatives inhibit this enzyme *in vivo*.

Thus, earlier findings of DAO inhibition based on indirect measurements are confirmed.[15–19] Furthermore it is shown that there exists no parallelism between the *in vivo* and the *in vitro* potency of DAO inhibitors. Thus, the quotient:

$$\frac{\text{final concentration for 50 per cent inhibition } \textit{in vitro} \times 100}{ED_{50} \textit{ in vivo}}$$

(Table 3) is high (1 or more) for iproniazid, N^1-*iso*nicotinyl-N^1 : N^2-di*iso*propylhydrazine, N^1 : N^2-di*iso*propylhydrazine and N^1 : N^2-di*iso*propylsemicarbazide, indicating a relatively stronger inhibition *in vivo* compared to *in vitro*. On the other hand the quotient is low for isoniazid, semicarbazide and aminoguanidine (0·18 and less) suggesting a relatively weaker effect *in vivo* compared to *in vitro*. The change of the

TABLE 3. COMPARISON OF 50 PER CENT INHIBITION OF DAO *in vitro* AND *in vivo* (values from Table 2)

Compounds	Quotient*
Iproniazid	1·3
Isoniazid	0·18
N^1-*iso*Nicotiynl-N^1 : N^2-di*iso*propyl	>52
*iso*Propylhydrazine	0·08
N^1 : N^2-Di*iso*propylhydrazine	1·0
N^1 : N^2-Di*iso*propylsemicarbazide	>66
Semicarbazide	0·01
Aminoguanidine	0·001

* Quotient $= \dfrac{\text{final concentration for 50 per cent inhibition } \textit{in vitro} \times 100}{ED_{50} \textit{ in vivo}}$

A small quotient indicated a relatively stronger potency *in vitro* than *in vivo* and vice versa.

relative potency of some compounds *in vivo* might be caused by metabolic alterations or a different degree of penetration to the reactive site of the enzyme. Concerning MAO inhibition there exists some evidence that the *iso*propylhydrazine moiety is the active part of the iproniazid molecule[3, 10] and that it possibly splits off *in vivo*.[34]

Inhibition of DAO *in vivo* by iproniazid lasts much shorter than that of MAO (Fig. 1).[8, 28, 35, 36] However, the inhibition of DAO similar to that on MAO[2, 28, 29] is hardly reversible by dialysis. Therefore the relatively quick recovery of DAO activity in rat intestine *in vivo* after administration of iproniazid might be due to rapid resynthesis of the blocked enzyme.

Iproniazid has some clinical actions which are less pronounced after application of isoniazid (e.g. antidepressive effect in mental disorders, hypotension, pain relief in angina pectoris). It has been assumed that these effects of iproniazid are related to MAO inhibition which *in vitro* as well as *in vivo* is more marked with iproniazid than with isoniazid. DAO has not been considered because the two compounds equally inhibit this enzyme *in vitro*. The present results show that iproniazid *in vivo* unlike *in vitro* is a markedly stronger DAO inhibitor than isoniazid*. Thus, DAO inhibition

* Since this manuscript was submitted, the following further results have been obtained: Comparing inhibition of DAO and MAO in the same organ (cat kidney) DAO *in vivo* was about twice as much inhibited ($P < 0{\cdot}05$) by iproniazid as MOA (K. F. GEY, W. P. BURKARD and A. PLETSCHER, *Helv. Physiol. Acta*, in press (1960)).

could possibly also be of importance for some clinical and pharmacological effects of iproniazid.

Typical substrates the metabolism of which might be altered after DAO inhibition are agmatine, cadaverine, putrescine, spermine, spermidine[37] but in most species probably not histamine.[20, 38, 39, 40] Until the physiological function of these diamines is better known, no definite comments can be made on the possible consequences of DAO inhibition.

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KINETICS OF PLASMA DRUG LEVELS AFTER SUSTAINED RELEASE DOSAGE

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Abstract—Equations giving plasma and urinary drug levels in terms of apparent first order absorption and elimination of drug, apparent volume of distribution of drug per kilogram, time, and release characteristics of sustained release preparations, are derived for the cases of oral administration of drug solution or a sustained release preparation. The equations are shown to describe the plasma levels obtained in dogs after administration of HT1479 (1-(*o*-methoxyphenyl)-4-(γ-methoxypropyl)-piperazine phosphate) as an oral solution or in sustained release tablets. The apparent first order constants for absorption and disappearance of drug from plasma were 2·0 hr^{-1} and 0·28 hr^{-1}, respectively, and the release constants for the Gradumet tablet were similar to those obtained *in vitro*.

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MATHEMATICAL equations giving plasma levels of drugs after oral administration, based on first order absorption and elimination rates, have been derived by Gehlen,[1] Teorell,[2] Butler, *et al.*,[3] Dost,[4] and Warter and Metais.[5] With the exception of those of Warter and Metais,[5] the equations are all of the same basic type described by Bray and White[6] for a sequence of two irreversible first order processes. There is evidence that, for some drugs, either absorption or elimination, or both, do follow apparent first order kinetics,[3, 5, 7, 8] and thus the mathematical treatment of these phenomena allow calculation of rate constants which adequately describe the system.

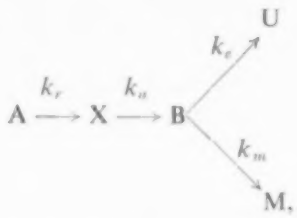
Sustained release preparations, a type of oral medication designed to liberate at least a fraction of the drug content in a continuous manner over a period of several hours, have been shown in some instances to release this drug in an exponential manner *in vitro*.[9] If the release of drug *in vivo* is also an apparent first order process, the kinetics of plasma drug levels should be described by an extension of the equation for plasma levels of drugs absorbed and eliminated by first order kinetics. This will be complicated by the release of a fraction of the dose essentially immediately, this fraction following the simpler two-sequence equation, but the complete system can be described by a single equation incorporating the parameters of release, absorption, and elimination.

A kinetic analysis of plasma drug levels is important to a clear understanding of the absorption and elimination of drugs and the effect of sustained release dosage forms. The mathematical treatment presented in this paper is straightforward and gives a sound basis on which to evaluate the effectiveness of delayed release of drug.

MATHEMATICAL DERIVATION

After administration of a sustained release preparation, it is assumed below that

drug will pass irreversibly through a series of compartments before it is eventually excreted or metabolized. The first compartment to consider is the sustained release preparation itself. From the preparation, drug is released into the gastrointestinal tract, and is then absorbed into the plasma and other tissues of the body. From the plasma the drug may be excreted into the urine or metabolized. These transformations can be shown in a flow diagram as follows:



where A indicates the sustained release preparation, X indicates the gastrointestinal tract, B the plasma and other tissues of the body, U the urine, and M the total metabolites in plasma, tissue or urine. In the derivation which follows, the amounts of drug in each compartment at any time t will be denoted by the small letters a, x, b and u for the compartments with the respective designations. Assuming that the rate of transfer from one compartment to the next is in each case proportional to the amount of drug in the preceding compartment, first order rate constants can be assigned as indicated above, k_r being the first order release constant, k_a the first order absorption constant, k_e the first order excretion constant, and k_m the first order metabolism constant.

For a sustained release preparation which releases at least a fraction of its drug content at a rate which is proportional to the amount of drug remaining in the tablet (a),

$$-\frac{da}{dt} = k_r a \tag{1}$$

expresses the rate of change of amount of drug remaining in the tablet. If the amount of drug in the tablet before administration is a_o, and the fraction f_r is released exponentially, equation (1) upon integration gives the amount of drug remaining in the tablet* at any time t as

$$a = a_o f_r e^{-k_r t} \tag{2}$$

The amount of released drug in the gastrointestinal tract at time zero for a sustained release preparation which releases a fraction (f_i) of its drug content immediately is $a_o f_i$. The amount of drug in this compartment at any time t is increased by continued release from the tablet and decreased by absorption into the plasma according to the differential equation

$$\frac{dx}{dt} = k_r a - k_a x \tag{3}$$

* Where a_r is the amount of drug released from the tablet at time t, it can be shown[9] that $a_r = a_o f_i + a_o f_r(1 - e^{-k_r t})$ where f_i is the fraction of the total drug released immediately.

or, substituting for a from equation (2)

$$\frac{dx}{dt} = a_o f_r k_r e^{-k_r t} - k_a x \tag{4}$$

Integration of equation (4), solving for the integration constant at $t = 0$, $x = a_o f_i$, gives

$$x = \frac{a_o f_r k_r}{(k_a - k_r)} (e^{-k_r t} - e^{-k_a t}) + a_o f_i e^{-k_a t} \tag{5}$$

At zero time the amount of drug in compartment B is zero, but the amount increases by absorption from the gastrointestinal tract and is decreased by excretion and/or metabolism according to the expression

$$\frac{db}{dt} = k_a x - k_e b - k_m b \tag{6}$$

or, after substitution for x from equation (5) and collecting terms,

$$\frac{db}{dt} = \frac{a_o f_r k_a k_r}{(k_a - k_r)} (e^{-k_r t} - e^{-k_a t}) + a_o f_i k_a e^{-k_a t} - (k_e + k_m) b \tag{7}$$

After integration, equation (7) gives

$$b = \frac{a_o f_r k_a k_r}{(k_a - k_r)(k_e + k_m - k_r)} (e^{-k_r t} - e^{-(k_e + k_m)t}) + \frac{a_o f_i k_a - \frac{a_o f_r k_a k_r}{(k_a - k_r)}}{(k_e + k_m - k_a)} (e^{-k_a t} - e^{-(k_e + k_m)t}) \tag{8}$$

Equation (8) is the desired expression for the amount of drug in the plasma and tissues (exclusive of drug in the gastrointestinal tract and urine) after administration of a sustained release preparation. If only plasma drug levels are to be obtained, no measure of individual values of k_e and k_m will be obtained, and these rate constants accounting for disappearance of drug from plasma can be combined into one parameter, k_d. If a_o is defined as the dose in mg/kg, b is mg of drug per kilogram body weight. Dividing the right side of equation (8) by the specific apparent volume of distribution,* V'_d, gives plasma concentration, $c = b/V'_d$. Incorporating these changes equation (8) becomes

$$c = \frac{a_o f_r k_a k_r}{V'_d(k_a - k_r)(k_d - k_r)} (e^{-k_r t} - e^{-k_d t}) + \frac{a_o f_i k_a - \frac{a_o f_r k_a k_r}{(k_a - k_r)}}{V'_d(k_d - k_a)} (e^{-k_a t} - e^{-k_d t}) \tag{9}$$

* Apparent volume of distribution[8, 10] has the dimension liters when the total dose is used. For doses in mg/kg, the volume of distribution is most readily obtained on a per kilogram basis, l/kg, and it is proposed that this be termed the specific apparent volume of distribution, V'_d defined as milligrams drug per kilogram body weight divided by plasma concentration in milligrams per liter. The definition should not be construed to exclude use of serum or whole blood, or the uniform substitution of weights other than in milligrams—this does not change the dimensions of V'_d from l/kg. Although the term has little physiological meaning when the value is greater than the total body water content per kilogram (indicating concentration in the extravascular spaces), values of V'_d in the range 0·1 to 0·7 indicate the available space in which the drug is distributed.

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If an oral solution of drug, or a tablet or capsule designed to release its drug content essentially immediately, is administered, the plasma drug levels can be obtained from equation (9) by noting that under these conditions $f_r = 0$ and $f_i = 1$. Thus equation (9) reduces to

$$c = \frac{a_o k_a}{V_d'(k_d - k_a)} (e^{-k_a t} - e^{-k_d t}) \tag{10}$$

which is the same form derived previously for this case.[1, 2, 4, 6]

The fraction of a_o in the urine as a function of time will be given by $du/dt = k_e b$. Substitution from equation (8), integration and rearranging gives

$$u = a_o(f_i + f_r)\frac{k_e}{(k_e + k_m)} - \frac{a_o f_r k_a k_e}{(k_a - k_r)(k_e + k_m - k_r)} \left(e^{-k_r t} - \frac{k_r}{(k_e + k_m)} e^{-(k_e + k_m)t}\right) - \frac{a_o f_i k_e - \frac{a_o f_r k_e k_r}{(k_a - k_r)}}{(k_e + k_m - k_a)} \left(e^{-k_a t} - \frac{k_a}{(k_e + k_m)} e^{-(k_e + k_m)t}\right) \tag{11}$$

For oral solution of drug ($f_i = 1$, $f_r = 0$) this reduces to

$$u = a_o \frac{k_e}{(k_e + k_m)} - \frac{a_o k_e}{(k_e + k_m - k_a)} \left(e^{-k_a t} - \frac{k_a}{(k_e + k_m)} e^{-(k_e + k_m)t}\right) \tag{12}$$

If $f_i + f_r = 1$ equations (11) and (12) both reduce at t equal to infinity to $u = a_o k_e/(k_e + k_m)$, indicating that the fraction of the drug excreted is determined by the relative rates of excretion and metabolism.

EXPERIMENTAL AND RESULTS

HT1479 (1-(*o*-methoxyphenyl)-4-(γ-methoxypropyl)-piperazine phosphate)* was orally administered to mongrel dogs as an aqueous solution and in the form of a plastic-matrix type sustained release preparation in tablet form (Gradumet†). Food but not water was withheld on the day of the experiment. The same six dogs were used with both dosage forms, at least a week elapsing between experiments using the same animal. HT1479 sustained release tablets, 75 mg, were administered on the basis of one per kilogram or fraction of a kilogram, the average dose actually received being 79·0 mg/kg. The oral solution of HT1479 was then calculated to be one-half the sustained release dose, or 39·5 mg/kg, administered by stomach tube as a solution containing 39·5 mg/ml, followed by 10 ml of water. Blood samples of 10 ml were drawn just prior to dosing and ½, 1, 1½, 2, 3, 4 and 5 hr after dosing. Plasma samples of 4 ml were analyzed for HT1479 by the methyl orange procedure of Brodie and Udenfriend.[11] The plasma levels of HT1479 and the standard deviations are given in Table 1.

The *in vitro* release of HT1479 from the sustained release tablet (lot no. 709-8933) was determined by placing six HT1479 sustained release tablets and 300 ml distilled

* Covered in U.S. pat. 2,891,063, assigned to Abbott Laboratories.
† Gradumet is the registered trademark of Abbott Laboratories.

TABLE 1. HT1479 FREE BASE PLASMA CONCENTRATIONS (UG/ML) IN DOGS

	Time hr	668*	1059	711	816	1067	1069	Average ± S.D.
Solution, 39·5 mgPO_4 salt/kg, orally	0·5	10·12	1·10	5·03	3·50	3·50	5·80	4·84 ± 3·05
	1·0	7·60	3·23	7·00	6·76	6·26	4·53	5·90 ± 1·67
	1·5	7·00	3·93	6·77	6·60	5·67	3·90	5·64 ± 1·41
	2·0	6·95	3·87	7·43	5·50	5·33	2·53	5·27 ± 1·84
	3·0	5·05	3·37	5·23	3·60	3·56	1·10	3·65 ± 1·49
	4·0	4·42	3·30	4·13	4·26	3·16	0·73	3·33 ± 1·38
	5·0	3·98	2·37	3·63	2·50	2·23	0·10	2·47 ± 1·36
Sustained release tablets, 79·0 mg/PO_4 salt/kg, orally	0·5	0·35	1·25	1·65	0·70	3·05	2·42	1·57 ± 1·02
	1·0	2·18	3·75	3·42	3·15	3·05	4·28	3·30 ± 0·71
	1·5	4·20	4·70	6·55	4·50	4·00	3·75	4·62 ± 1·01
	2·0	4·55	4·20	7·08	4·95	4·13	3·84	4·79 ± 1·18
	3·0	5·98	5·68	7·08	5·20	7·25	4·88	6·01 ± 0·97
	4·0	6·25	4·95	6·88	6·10	6·53	4·50	5·87 ± 0·94
	5·0	5·45	4·25	5·78	5·03	6·36	4·12	5·16 ± 0·88

* Dog numbers.

water in a 600-ml wide-mouth bottle, rolling at 100 rpm at room temperature and taking, at various intervals, 10-ml aliquots for assay. The amount of drug released was calculated by correcting for the volume of solution present when the aliquot was removed and for the amount of HT1479 removed in previous aliquots. The per cent of drug released from the sustained release tablet was 20·4, 32·4, 68·0 and 88·0 at $\frac{1}{4}$, 1, 4 and 8 hr. Data are plotted semi-logarithmically in Fig. 1 with the least squares line calculated from the data.

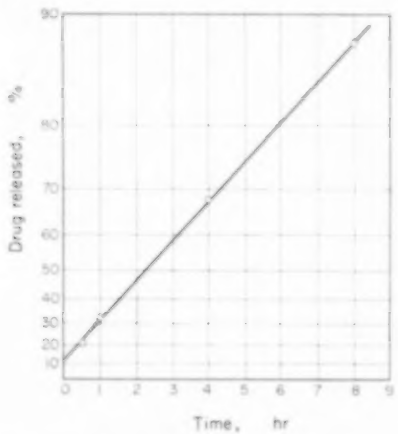

FIG. 1. *In vitro* release curve for 75 mg HT1479 sustained release tablets, with the least squares line from which the values $k_r = 0{\cdot}25\ hr^{-1}$, $f_t = 0{\cdot}12$ and $f_r = 0{\cdot}88$ are calculated.

DISCUSSION

The interpretation of plasma drug levels, following oral administration of a drug solution, in terms of two simultaneous apparent first order processes, has several advantages. One is that a smooth theoretical curve can be drawn through the experimental points, partly eliminating the subjective factor in fitting a curve to data. Extrapolation of plasma levels to times later than that for which experimental values

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are available is also facilitated, and allows this to be done based on the data in the earlier time period. In addition to these graphic advantages, measures of the rates of absorption of the drug from the gastrointestinal tract and disappearance of the drug from plasma are obtained, and the order of the kinetics which describes the system is verified.

The apparent first order absorption and disappearance rates are calculated on the assumption that these processes are occurring in an apparent first order manner. Although for some drugs this has been demonstrated,[3, 5, 7, 8] the decision for a given drug in a given species must be based on whether or not the curve adequately fits the data. Fig. 2 shows that plasma levels of HT1479 following oral administration of

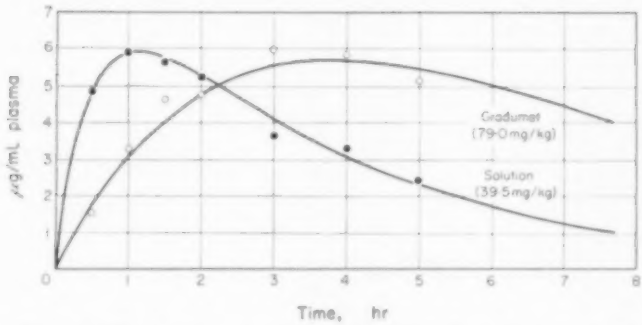

FIG. 2. Average plasma HT1479 free base concentrations from six dogs after administration of 39·5 mg PO_4 salt/kg as solution (●) and 79·0 mg PO_4 salt/kg as sustained release tablets (○). The curves are calculated according to equation (10) for solution with $a_o = 28{\cdot}8$ mg/kg and equation (9) for sustained release tablets with $a_o = 57{\cdot}6$ mg/kg, $k_r = 0{\cdot}25$, $f_a = 0{\cdot}12$, $f_r = 0{\cdot}88$, and the values in both equations of $k_a = 2{\cdot}0\ hr^{-1}$, $k_d = 0{\cdot}28\ hr^{-1}$ and $V'_d = 3{\cdot}52$ l/kg.

the solution to dogs are adequately described by a curve calculated from equation (10), and thus absorption and disappearance of HT1479 follow apparent first order kinetics. The apparent first order absorption constant, k_a, is 2·0 hr^{-1}, and the apparent first order constant which describes disappearance of drug from the plasma, k_d, is 0·28 hr^{-1}.

Disappearance of drug from the plasma is a complex phenomenon involving metabolism, excretion, and equilibration with tissues. Assigning a first order constant to the overall process assumes that metabolism and excretion are both first order processes or that one, if it is sufficiently greater in magnitude such that the other is negligible, is first order. On the basis of these assumptions no data are obtained to allow a measure of individual rates of metabolism or excretion. The steady state process of equilibration of drug between plasma and tissue is assumed to be rapid and therefore would not appreciably affect the values of k_a and k_d obtained. If equilibration were not sufficiently rapid to be neglected, it would influence the more rapid process more. This would be absorption in most cases, as it is for HT1479. The contribution from this source to the absorption constant is not readily determined, and this effect is usually neglected.

Calculation from equation (10) of the curve for plasma levels of HT1479 from solution, as shown in Fig. 2, was first done substituting the dose, 28·8 mg free base/kg, for a_o. By successive approximation the values of k_a, k_d and V'_d with time in hours

were determined to give the best fit to the data. Although the values of k_a, k_d and V_d' are mutually dependent to some degree, k_a primarily determines the slope of the ascending portion of the curve, k_d the slope of the descending portion, and V_d' the height of the whole curve. If one or two experimental points do not fall as close to the calculated curve as the others, but the curve closely approximates the remaining points, the value of the volume of distribution per kilogram, V_d', determined may be different than that calculated from the average theoretical amounts of drug present divided by the experimental plasma levels. Because of the excellent agreement between the theoretical and experimental plasma levels obtained with HT1479 solution, the value of the specific volume of distribution (volume of distribution per kilogram) used to obtain the curve shown in Fig. 2, 3·52 l/kg, is close to the average of the calculated values, 3·53 l/kg (see Table 2).

TABLE 2. SPECIFIC VOLUME OF DISTRIBUTION OF HT1479

Time, (hr)	Spec. vol. Dist. (l/kg)*	
	Solution	Sustained release tablets
0·5	3·47	3·92
1·0	3·52	3·30
1·5	3·61	3·11
2·0	3·52	3·52
3·0	3·94	3·24
4·0	3·28	3·40
5·0	3·34	3·72
average ± S.D.	3·53 ± 0·21	3·46 ± 0·28

* Calculated as theoretical mg drug/kg (from $b = cV_d'$ and equations (9) and (10)) divided by average experimental plasma levels (mg/l).

Absorption of drug and disappearance of drug from the plasma should be governed by the same rate constants when HT1479 is administered in sustained release form, with the possible exception that the absorption rate constant may vary with the level of the gastrointestinal tract at which absorption takes place. Calculation of the theoretical plasma curve according to equation (9) for plasma levels following administration of the sustained release tablets was therefore based on the same values of k_a and k_d used to calculate the plasma curve for the solution experiment. Values of the apparent first order release constant (k_r) and f_i and f_r were the same as calculated from the least squares line fit to the *in vitro* release curve of the sustained release tablet[9] (see Fig. 1). The volume of distribution per kilogram used in the calculation was the same as was used in the calculation for the plasma curve following administration of HT1479 solution (3·52 l k/g). The dose, a_o, was 57·6 mg of the free base per kilogram (equivalent to 79·0 mg of the phosphate salt per kilogram). The curve obtained is given in Fig. 2 with the average experimental points.

The values for the specific volume of distribution calculated from theoretical body drug content and experimental plasma concentrations following administration of sustained release tablets are given in Table 2. The average value, 3·46 l /kg, is not

significantly different from the value used in calculation of the curve (3·52 l/kg). The height of the calculated curve is close to the experimental plasma levels, as shown by the average volume of distribution, indicating that the drug is available from the sustained release tablet and is absorbed from the gastrointestinal tract at the levels where it is released.

The calculated curve fits the experimental points with adequate precision to suggest that the assumptions on which equation (9) is based are operative. The release characteristics of the sustained release tablet *in vivo* are therefore essentially the same as determined *in vitro* in aqueous solution. Changing the value of k_r to 0·28 hr^{-1} in equation (9) gives a curve* which fits the data as well as $k_r = 0{\cdot}25\ hr^{-1}$, but values outside the range 0·25–0·28 hr^{-1} lead to progressively larger values of the sum of the squares of the deviations.

Excretion studies should provide estimates of absorption and disappearance of drug from the plasma using equation (11) or (12). In addition, an estimate of the excretion rate and metabolism rate may be obtained from the asymtotic value of the cumulative excretion curve. The accuracy of the values of the absorption rate, and also of release characteristics of sustained release preparations, will decrease, however, because the system used to measure these values is one compartment further away from these phenomena than when using plasma levels.

* When the rate constants k_r and k_d are equal equation (9) reduces[4] to

$$c = \frac{a_0 f_r k_a k t}{V_d' (k_a - k)} e^{-kt} + \frac{a_0 f_r k_a - \dfrac{a_0 f_r k_a k}{(k_a - k)}}{V_d' (k_a - k)} (e^{-kt} - e^{-k_a t}), \text{ where } k_r = k_d = k.$$

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INVESTIGATION ON THE MODE OF ACTION OF *CYCLO*SERINE UPON PROTEIN SYNTHESIS OF *E.COLI* AND ANIMAL CELLS

II—ACTION OF L-*CYCLO*SERINE ON PROTEIN METABOLISM OF ALANINE AND ON ENZYMIC PREPARATIONS

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Abstract—The action exerted by L-*cyclo*serine on protein metabolism of alanine in animal and bacterial cells has been investigated. High concentrations of L-*cyclo*serine inhibit the incorporation of alanine-^{14}C into proteins while low concentrations increase it. It has also been demonstrated that the L-isomer of *cyclo*serine, contrary to the D-isomer, causes a marked inhibition on the enzymic transamination which involves the formation of L-alanine.

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INTRODUCTION

IT IS known that the L- and D-enantiomorphs of *cyclo*serine possess different antibacterial activities[1] and pharmacological properties.[2–5] In our previous biochemica experiments[6] we observed that L-*cyclo*serine, contrary to the D-isomer, increases alanine-^{14}C incorporation into proteins of *E. coli*.

The present paper deals with the influence of L-*cyclo*serine on protein metabolism of alanine and with the transaminating activity of bacterial suspensions and rat liver homogenates.

MATERIALS AND METHODS

A. *Strains, preparation of extracts, chemicals*

(a) *E. coli* 147, a thymine and phenylalanine-requiring strain, isolated in our laboratory, was grown on a Davis[7] medium containing limited amounts of thymine, and was harvested for radioactive experiments when growth was stopped as a consequence of thymine exhaustion. The Davis synthetic medium was supplemented with 25 μg/ml of Difco casein hydrolysate and 75 μg/ml of DL-phenylalanine.[6]

(b) Acetone-dried cells of *E. coli* B and 147 were prepared according to Umbarger method.[8] For enzymatic experiments, the packed cells were suspended in 10 vol of cold potassium phosphate buffer solution (0·1 M; pH 7·4) previously homogenized at 0 °C in a Potter apparatus for 5 min.

(c) Hepatoma ascites AH130 cells from albino rats were withdrawn 7 days after inoculation. The strain was kindly supplied by Professor T. Yoshida. Cells were washed with isotonic solution, suspended in two volumes of Krebs–Ringer-HCO_3 synthetic medium at pH 7·4 and incubated at 37 °C for 2 hr, for radioactive experiments.

(d) Liver, brain and hepatoma ascites AH130 cell homogenates from Wistar rats were prepared in a glass homogenizer with a Teflon pestle at 1000 rev/min at 0 °C for

1 min in 10 vol of a 0·25 M sucrose solution. The suspensions were incubated at 37 °C for 1 hr, with shaking, for radioactive experiments.

(e) Rat liver homogenates were prepared in a glass homogenizer with a Teflon pestle, using 20 ml of ice-cold potassium phosphate buffer (0·1 M; pH 7·4) per g of freshly dissected tissue, for enzymatic experiments.

(f) Supernatant fractions from liver and hepatoma ascites AH130 homogenate, according to (d), were obtained by centrifuging at 15,000 *g* for 10 min at 0°C, for radioactive experiments.

(g) A suspension of microsomes and pH 5 enzymes was prepared in medium A[9] from the liver homogenates mentioned above.

(h) The D-isomer of *cyclo*serine was isolated from a culture of *St. gariphalus* obtained in the Farmitalia Antibiotic Department of Settimo Torinese. The L-isomer was obtained by the Farmitalia Chemical Department of Settimo Torinese, after isolation from the synthetic racemate.

(i) Pyridoxal-phosphate was supplied by La Roche; ^{14}C-amino acids were obtained from the Radiochemical Centre of Amersham (England).

B. *Biochemical methods*

(1) The preliminary estimation of the protein content of the subcellular fractions (d), (f) and (g) was performed according to Kunitz.[10]

(2) *Protein-*^{14}C *extractions.* Proteins were extracted according to Rabinovitz method.[11] Protein metabolism of the samples incubated with radioactive aminoacids and *cyclo*serine, was blocked by addition of trichloracetic acid (TCA) to give a 5 per cent final concentration. The protein fraction was purified by washing TCA insoluble material twice with cold, twice with hot, (at 95°C for 15 min) and again twice with cold 5 per cent TCA; then once with ethanol and three times with hot ethanol–ether 3 : 1 (at 65 °C for 5 min). Finally the protein fraction was completely dried with ether.

(3) *Radioactivity measurements.* When the amount of material was scarce, it was suspended in chloroform and transferred into polyethylene planchets, then the solvent was evaporated. In any case the radioactivity of the experimental samples was measured using 1 cm^2 polyethylene planchets and thin mica window Tracerlab G.–M. tubes (TGC2) and employing, when necessary, a self-absorption correction factor.

(4) *Determination of transaminase activity.* The transaminase activity of liver homogenates and *E. coli* cells suspensions (strain B and strain 147), in the presence and absence of L-*cyclo*serine, was tested according to the following reactions:

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyruvate} + \text{L-glutamate} \qquad (1)$$

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxalacetate} + \text{L-glutamate} \qquad (2)$$

Pyruvate was analysed as 2 : 4-dinitrophenyl-hydrazone while oxalacetate was previously converted to pyruvate.[12] Rat liver homogenates were prepared in potassium phosphate buffer according to (e). *E. coli* cell homogenates were prepared in the buffer solution according to (b). The *E. coli* buffered suspension was not diluted further when used in testing L-alanine reaction (equation (1)) but it was diluted when used to test the L-aspartate reaction (equation (2)). A five fold dilution was employed in the experiments with strain 147 and an eight fold dilution with strain B. The sample was prepared as follows: to 0·1 ml of enzymic preparation were added 0·9 ml of potassium

phosphate buffer (0·1 M; pH 7·4) containing 0·05 mM of L-aminoacid, 0·02 mM of ketoacid, 100 μg of pyridoxal phosphate, and some of either one or the other of the two isomers of *cyclo*serine when this substance was used. The final volume of the sample was 1 ml. The enzymic processes were blocked by addition of 10 % TCA. Rat liver homogenates were incubated at 37 °C for 10 min. For *E. coli* dried cells of the two *E. coli* strains, which exhibit a weak transaminating action towards L-alanine and a strong one towards L-aspartate, we had to use two different concentrations, as mentioned. In addition, for alanine reaction the *E. coli* 147 cell suspensions

Table 1. Action of LCS on the incorporation of algal protein hydrolysate-^{14}C and alanine-^{14}C into proteins of *E. coli* 147 and rat hepatoma ascites AH130

Labelled precursor	Cell system*	Controls	LCS 0·1 μM/ml	LCS 1 μM/ml
Algal protein hydrolysate	*E. coli* 147	7622	7297	—
Algal protein hydrolysate	AH 130	570	563	473
DL-Alanine C14	*E. coli* 147	3417	5865	2770
L-Alanine C14	AH 130	575	2370	1867

Values are given as counts/min per mg of protein.

* The final volume of each sample of bacterial cell suspension was 10 ml or 20 ml according to the experiment; in any case the bacterial suspension showed an optical density of 1000 at 700 mμ. Samples were incubated for 40 min without shaking.

The final volume of each sample of hepatoma ascites cell suspension was 2 ml and samples contained 1μc of either L-alanine-^{14}C or algal protein-^{14}C hydrolysate. They were incubated for 2 hr with shaking.

Table 2. Action of LCS on the incorporation of L-alanine-^{14}C-G into proteins of mammalian subcellular system

Labelled precursor: L-alanine-^{14}C-G (μc/ml)	System*	Controls	LCS 0·1 μM/ml	LCS 1 μM/ml
1·9	Rat liver homogenate	2·34	3·60	2·53
1·9	Rat liver homogenate + α-ketoglutarate	5·42	3·98	4·19
3·5	Rat brain homogenate	4·34	4·13	3·19
1·9	Supernatant 15,000 *g* of rat hepatoma ascites AH 130	2·57	2·00	3·40
1·9	Supernatant 15,000 *g* of rat liver	4·94	3·98	3·93
1·2	Microsomes + EpH5 of rat liver	10·34	11·59	13·83

Values are given as counts/min per mg of protein.

* Each sample of liver and brain homogenate contained in a final volume of 2 ml : 1 ml of homogenate preparation in 0·25 M sucrose; 0·2 ml of a 0·25 M sucrose, 0·016 M ATP, 0·2 M α-ketoglutarate,[13] and 0·05 M $MgCl_2$ solution; 0·5 ml of potassium phosphate buffer pH 7·4 (0·1 M); 0·3 ml of a 0·25 M sucrose solution containing L-alanine-^{14}C (specific activity 5 μc/μM).

Each sample of 15,000 *g* supernatant fraction contained, in a final volume of 2 ml of medium A: 20 μM of HDP, 2 μM of ATP, 20 mg of supernatant fraction proteins, 3·8 μc of L-alanine-^{14}C.

Each sample of microsomes + E pH 5 suspension contained, in a final volume of 1 ml of medium A: 1 μM of ATP, 0·5 μM of GTP, 10 μM of PEP, 20 μg of PEP-kinase, 1·2 μc of L-alanine-^{14}C, 10 mg of microsomal proteins and 2·5 mg of E-pH 5 proteins.

All samples were incubated for 1 hr at 37 °C with shaking.

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were incubated for 4–5 hr and the *E. coli* B cell suspension for 60–90 min. All samples were incubated for 10 min when the reaction contained L-aspartate.

EXPERIMENTAL

Effect of L-cyclo*serine on alanine-*^{14}C *incorporation into proteins*

When whole cells of *E. coli* 147 and rat hepatoma ascites AH130 are employed and when the protein precursor is α-alanine-^{14}C, L-*cyclo*serine, at the concentration of 0·1 μM/ml, causes an increase in the incorporation of labelled aminoacids into cell proteins. If α-alanine-^{14}C is replaced by a mixture of ^{14}C-amino acids from an algal protein hydrolysate, there is no significant change.

On the other hand, when the incorporating system consists of rat subcellular fractions, the stimulating action of L-*cyclo*serine is doubtful. (Table 2).

TABLE 3. PERCENTAGE VARIATIONS OF THE SPECIFIC ACTIVITY OF CELL PROTEINS OF *E. coli* 147 INCUBATED WITH DL-ALANINE-^{14}C IN THE PRESENCE OF DCS, LCS, PLP AND A MIXTURE OF LCS AND PLP

Experiment no. 1		Experiment no. 2	
Additives (μM/ml)	% Variations of protein specific activity as compared to the control	Additives (μM/ml)	% Variation of protein specific activity as compared to the control
LCS 0·04	+ 7·8	LCS 0·04	+ 33·2
—		LCS 0·10	+ 47·5
—		LCS 0·20	+ 47·0
PLP 0·04	− 23·6	PLP 0·04	− 14·2
PLP 0·12	− 30·0	—	
PLP 0·32	− 24·7	—	
LCS 0·04 PLP 0·04	− 7·5	LCS 0·04 PLP 0·04	+ 21·2
LCS 0·04 PLP 0·12	− 6·5	— —	
LCS 0·04 PLP 0·32	− 11·7	— —	
— —		LCS 0·10 PLP 0·04	+ 33·2
— —		LCS 0·20 PLP 0·04	+ 33·4
DCS 0·20	− 27·0		
DCS 0·20 PLP 0·04	− 47·5		
DCS 0·20 PLP 0·12	− 49·5		
DCS 0·20 PLP 0·32	− 51·0		

* PLP = Pyridoxalphosphate.

Table 3 reports the percentage variations occurring in the specific activity of *E. coli* 147 proteins when bacterial suspensions are incubated, in the presence of DL-alanine-^{14}C, either with L-*cyclo*serine, or pyridoxalphosphate or a mixture of the two.

Influence of L-cyclo*serine and* D-cyclo*serine on the transamination by enzymic systems*

Rat liver homogenates. When the transaminating activity of rat liver homogenate is tested in the reaction L-alanine + α-ketoglutarate ⇌ pyruvate + glutamate, D-*cyclo*serine does not show any inhibiting action up to a concentration of 1 μM/ml while the inhibiting action of L-*cyclo*serine is already strong at concentrations of 0·02 μM/ml and complete at concentrations of 0·20 μM/ml.

TABLE 4. ACTION OF DCS AND LCS ON THE FORMATION OF PYRUVATE FROM L-ALANINE + α-KETOGLUTARATE IN THE PRESENCE OF RAT LIVER HOMOGENATE

DCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of pyruvate formation	LCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of pyruvate formation
1·00	0·04	2·25	0·02	0·04	54·00
1·00	0·40	7·00	0·02	0·40	57·50
2·00	0·04	3·40	0·20	0·04	98·00
2·00	0·40	2·00	0·20	0·40	98·50

When the reaction L-aspartate + α-ketoglutarate ⇌ oxalacetate + glutamate is used, both D- and L-*cyclo*serine, in concentrations ranging from 0·02 μM/ml to 0·2 μM/ml, show only a slight inhibiting action.

TABLE 5. ACTION OF DCS AND LCS ON OXALACETATE FORMATION FROM L-ASPARTATE + α-KETOGLUTARATE IN THE PRESENCE OF RAT LIVER HOMOGENATE

DCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxyalacetate formation	LCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxalacetate formation
0·02	0·04	2·50	0·02	0·04	2·00
0·02	0·40	11·50	0·02	0·40	14·00
0·20	0·04	10·00	0·20	0·04	9·30
0·20	0·40	16·50	0·20	0·40	16·00
2·00	0·04	43·40			

E. coli cells. When the transaminating activity of a suspension of *E. coli* dried cell is tested on the reaction L-alanine + α-ketoglutarate ⇌ pyruvate + glutamate, D-*cyclo*serine in concentrations up to 0·2 μM/ml has scarcely any effect on the formation of pyruvate, while L-*cyclo*serine displays an inhibiting action even stronger than that observed with liver homogenate.

The enzymic preparations from *E. coli*, incubated at 37 °C for a prolonged time, form pyruvate even in the absence of amino or ketoacids. The pyruvate formed in this case is not affected by addition of 1 μM/ml of D- or L-*cyclo*serine and therefore is ascribed to metabolic pathways not involving alanine transamination. The inhibition of bacterial alanine transamination by L-*cyclo*serine was determined by subtracting

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the amount of pyruvate produced in the "blank" from that produced in the samples incubated with alanine. The percentage inhibition of the transamination L-alanine ⇌ glutamate exerted by *cyclo*serine on the enzymic preparation of *E. coli* 147 and B, respectively, are reported in Tables 6 and 7.

TABLE 6. ACTION OF DCS AND LCS ON PYRUVATE FORMATION FROM L-ALANINE + α-KETOGLUTARATE IN THE PRESENCE OF THE *E. coli* 147 SUSPENSION

DCS + PLP (μM/ml)		% Inhibition of pyruvate formation	LCS + PLP (μM/ml)		% Inhibition of pyruvate formation
0·20	0·04	21·00	0·02	0·04	88·00
0·20	0·40	12·00	0·02	0·40	97·50
1·00	0·04	58·00	0·20	0·04	96·50
1·00	0·40	41·00	0·20	0·40	100·00
			1·00	0·04	90·00
			1·00	0·40	100·00

TABLE 7. ACTION OF DCS AND LCS ON PYRUVATE FORMATION FROM L-ALANINE + α-KETOGLUTARATE IN THE PRESENCE OF THE *E. coli* B SUSPENSION

DCS + PLP (μM/ml)		% Inhibition of pyruvate formation	LCS + PLP (μM/ml)		% Inhibition of pyruvate formation
0·20	0·04	10·00	0·02	0·04	63·00
0·20	0·40	39·00	0·02	0·40	91·50
			0·20	0·04	93·20
			0·20	0·40	93·20

When the test reaction is L-aspartate + α-ketoglutarate ⇌ oxalacetate + glutamate, neither D- nor L-*cyclo*serine show any inhibiting action in concentrations up to 0·20 μM/ml. In concentrations of 1 μM/ml their inhibition values are almost similar. The percentage inhibition values of L-*cyclo*serine on oxalacetate formation by enzymic preparations of *E. coli* 147 and B, respectively, are reported in Tables 8 and 9.

TABLE 8. ACTION OF DCS AND LCS ON OXALACETATE FORMATION FROM ASPARTATE + α-KETOGLUTARATE IN THE PRESENCE OF THE *E. coli* 147 SUSPENSION

DCS + PLP (μM/ml)		% Inhibition of oxalacetate formation	LCS + PLP (μM/ml)		% Inhibition of oxalacetate formation
0·02	0·04	0	0·02	0·04	0
0·02	0·40	0	0·02	0·40	0
0·20	0·04	0	0·20	0·04	0
0·20	0·40	0	0·20	0·40	0
1·00	0·04	30·00	1·00	0·04	46·00
1·00	0·40	30·00	1·00	0·40	44·00

Table 9. Action of DCS and LCS on the oxalacetate formation from aspartate + α-ketoglutarate in the presence of the *E. coli* B suspension

DCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxalacetate formation	LCS (μM/ml)	+ PLP (μM/ml)	% Inhibition of oxalacetate formation
0·02	10	0	0·02	0·04	2·50
0·02	100	0	0·02	0·40	0
0·20	10	3·50	0·20	0·04	0
0·20	100	0	0·20	0·40	0
1·00	10	20·00	1·00	0·04	32·00
1·00	100	5·00	1·00	0·40	13·00

DISCUSSION

The L-isomer of *cyclo*serine, as well as the D-isomer, does not seem to affect the incorporation into cell proteins of ^{14}C amino acids from algal protein hydrolysate. On the other hand, if we use alanine-^{14}C instead of an amino acid-^{14}C mixture *cyclo*serine exerts a remarkable action on the incorporation of this amino acid into cell proteins. In fact, low concentrations of L-*cyclo*serine increase the incorporation of this amino acid into protein while high concentrations decrease it.

Experiments carried out with L-*cyclo*serine and pyridoxalphosphate showed that these substances promote opposite effects on the incorporation of DL-alanine-^{14}C into protein of *E. coli* 147.

The depressing activity of pyridoxalphosphate, upon DL-alanine-^{14}C incorporation, may be ascribed to the property of this coenzyme to accelerate the biosynthesis of alanine, as it is better observed in vitamin B_6 requiring micro-organisms.[14]

Considering the antibacterial properties of L-*cyclo*serine as well as its inhibiting action on alanine ⇌ ketoglutarate transamination, the increase in protein radioactivity induced by L-*cyclo*serine could be considered as the effect of the inhibition exerted by this drug on the synthesis of alanine.

From the radioactive data (Table 3) we wonder whether a possible active opposition does occur between pyridoxalphosphate and L-*cyclo*serine towards some enzymic steps of alanine metabolism, even if the results of the first experiment of Table 3 would support this possiblity.

From this experiment it is apparent that the actions of pyridoxal phosphate and the D-isomer of *cyclo*serine do not exclude each other. In any case, assays performed on the influence of *cyclo*serine upon the transaminating activity of enzymic preparations show clearly that L-*cyclo*serine, more than the D-isomer, inhibits the transaminating reactions and displays a characteristic specificity towards the substrates of enzymic reactions. Therefore it does not affect the formation of oxalacetate from L-aspartate and α-ketoglutarate even when it is used at the same concentrations which inhibit strongly the formation of pyruvate from L-alanine and α-ketoglutarate. On the other hand, D-*cyclo*serine inhibits only partially the transaminating system when used in concentrations fifty times higher than those required for a complete inhibition by L-*cyclo*serine. It is worth noticing that L-*cyclo*serine causes the same inhibition when supplemented either with 10 μg/ml or 100 μg/ml of pyridoxalphosphate. This seems to exclude the occurrence of a competitive antagonism between L-*cyclo*serine and pyridoxalphosphate for the same transaminating enzymes.

The stereospecificity of the inhibiting action of L-*cyclo*serine (only L-*cyclo*serine inhibits the transaminating reaction of L-alanine) would rather suggest the presence of a competition, between L-*cyclo*serine and L-alanine, for the same transaminating enzyme, though previous works showed that the inhibiting action of L-*cyclo*serine on *E. coli* growth is antagonized, but not competitively, by L-alanine.[1]

In any case it is clear that the enantiomorphs of *cyclo*serine, even if possessing a common antibacterial activity, behave in a quite different way.

The D-isomer, indeed, interferes selectively with the mechanism involved in D-alanine incorporation into macromolecular complexes of the bacterial cell wall, and slightly affects the biosynthetic pathway of alanine formation, such as glutamate ⇌ pyruvate transamination. Moreover, D-*cyclo*serine does not exhibit any action on L-alanine-^{14}C incorporation into proteins of mammalian cells. On the other hand, L-isomer specifically inhibits a transaminating reaction which forms L-alanine either in bacterial or in mammalian cells. This is also significant in regard to the pharmacological properties of L-*cyclo*serine.

It is doubtful whether the data concerning the effect of L-*cyclo*serine on L-alanine-^{14}C incorporation into proteins of subcellular fractions are significative, because of the too weak extent of L-alanine-^{14}C incorporation into protein, observed in our experiments (Table 2).

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VARIATIONS IN RAT LIVER CALCIUM AND MAGNESIUM*

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Abstract—Significantly higher magnesium concentrations were found in the livers of young, healthy, female rats than in the livers of normal males of the same age. The calcium concentration in the liver from femae rats also tended to be higher than for males. Rats given thioacetamide or carbon tetrachloride had liver calcium concentrations up to sixty times normal values. Necrotic liver produced by yellow phosphorus administration had about three times the calcium concentration of normal liver. Fatty infiltration induced by choline deficiency produced no change in the calcium level of the liver. The magnesium concentration in the liver was affected to a far less extent by these treatments than the calcium.

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INTRODUCTION

IN 1956, Gallagher, *et al.*[1] observed that the liver calcium concentration of adult male rats showed increases of up to fortyfold after one subcutaneous injection of thioacetamide. Subsequently, Thiers and Reynolds[2] have indicated that carbon tetrachloride administration increased the Ca level of rat liver mitochondria. These results indicated that liver damage might result in increases in liver calcium concentration.

It was thought that these increases in Ca concentration might occur in all types of liver damage. In order to test the generality of this phenomenon and the specificity of the cell membrane to the increases in Ca transport into the cell, both the Ca and Mg levels of rat liver in various types of liver damage have been examined.

METHODS

Animals. Young adult male rats, unless otherwise indicated, were used. They were fed commercial rat cubes both before and during the administration of the hepatic toxins, except for the animals made choline deficient. These animals received a diet described by Kratzing and Windrum.[3] Some choline deficient animals received ethyl trichloroacetate which was injected subcutaneously at 0·075 ml/100 g body weight once daily for 5 days. Animals were killed by decapitation, the livers removed, weighed and analysed.

Toxic agents. Yellow phosphorus in peanut oil (5 mg P/ml) was given by intraperitoneal injections. Carbon tetrachloride was similarly injected as a 50 per cent solution

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in peanut oil. Thioacetamide was injected subcutaneously as a 2 per cent aqueous solution.

Analytical procedures. Calcium was determined by titration with ethylenediamine-tetra-acetic acid (EDTA) using murexide as indicator,[4] and the total calcium and magnesium by titration with EDTA, using Eriochrome Black T as indicator after phosphate had been removed by precipitation with morpholine nitrate. By this method calcium and magnesium have been conveniently estimated with a maximum error of 5 per cent.[5]

Nitrogen was determined by the method described by Francis *et al.*[6]

RESULTS AND DISCUSSION

Normal liver

Livers from sixteen young male rats and sixteen female rats of the same age were analysed for calcium and magnesium. The values are given in Table 1. The results showed that there was no difference in the calcium content per gramme of wet liver between male and female animals. However, livers from female rats had significantly greater amounts of magnesium per g of wet liver ($P<0{\cdot}001$). When the results were expressed in micromoles per gramme of liver nitrogen (μmoles/g) the females had significantly higher values for both calcium ($P < 0{\cdot}05$) and magnesium ($P < 0{\cdot}001$). Variations in the level of calcium in the liver occurred in both sexes. This may have been due to occluded blood although Thiers and Vallee[7] also found a big variation in the calcium content of rat liver even after the livers had been perfused. The concentration of magnesium in the liver was more constant than that of the calcium.

The values obtained for both the calcium and magnesium levels in normal liver are in agreement with those obtained by Thiers and Vallee[7] and Griswold and Pace[8], although neither group reported any difference made by the sex of the rats.

The ratio of liver to body weight in males (4·4 per cent) was greater than in females (3·8 per cent); this sex difference was highly significant ($P < 0{\cdot}001$).

This type of sex differentiation in growth has not been reported previously (see Webster, *et al.*[9]) and may be a characteristic of our colony of rats.

Damaged liver

Liver toxins can be divided into two main classes; those which primarily damage the centrilobular area, and those which first affect the peripheral portions of the liver lobule.[10] With the exception of yellow phosphorus, the toxins used produced centrilobular damage. The changes in liver calcium and magnesium concentrations produced by these agents are shown in Table 1.

Choline deficiency

Macroscopically the livers appeared very fatty. In wet liver the calcium level was not significantly greater than that found in normal animals, but there was a greater concentration of calcium in the choline-deficient livers when expressed on a nitrogen basis ($P < 0{\cdot}01$). In these choline-deficient animals a small decrease in the magnesium concentration of wet liver and an increase in the magnesium content per gramme of nitrogen occurred. It is considered that these changes are non-significant and are a consequence of the presence of fat in these livers. Ethyl trichloroacetate has been found by Kratzing and Windrum[3,11], to remove the excess lipid which accumulates

TABLE 1. THE CALCIUM AND MAGNESIUM CONCENTRATION IN DAMAGED LIVER

Treatment	Dosage	Sex	No. of animals	Mean body wt. (g)	Ca concentration		Mg concentration	
					μ moles/g wet liver	μ moles/g N	μ moles/g wet liver	μ moles/g N
untreated normal	—	M	16	89	0·76 ± 0·06*	23·6 ± 1·9	9·18 ± 0·22	289 ± 7
untreated normal	—	F	16	77	0·91 ± 0·08	33·0 ± 2·9	10·20 ± 0·10	360 ± 13
choline deficiency	3½ weeks deficiency	M	6	95	0·86 ± 0·10	33·6 ± 3·2	8·61 ± 0·28	341 ± 8
choline deficiency + ethyl trichloroacetate	5 × 0·075 ml/100 g body weight	M	5	79	0·73 ± 0·17	26·2 ± —	9·24 ± 0·23	321 ± —
yellow phosphorus	1 × 0·5 mg	M	6	Ca 90	0·80 ± 0·12	28·3 ± 4·1	8·62 ± 0·45	306 ± 16
	4 × 0·1 mg	M	5	Ca 100	1·98 ± 0·37	75·9 ± 12·4	7·83 ± 0·59	301 ± 15
carbon tetrachloride	3 × 0·05 ml	M	10	99	5·54 ± 2·6	226 ± 108	8·48 ± 0·18	334 ± 9
	3 × 0·2 ml	M	4	146	30·0 ± 7·3	1379 ± 341	8·20 ± 0·76	369 ± 31
thioacetamide	1 × 20 mg/100 g body weight	M	6	137	45·0 ± 2·5	1716 ± 95	13·43 ± 0·63	511 ± 20

* Standard error of mean.

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in the liver of choline-deficient rats. A group of choline-deficient rats was given five daily doses of ethyl trichloroacetate at a dosage which was previously found effective in clearing the liver of excess lipid. At death the livers of these rats had a normal appearance with no excess lipid. The calcium and magnesium concentrations in the liver were found to be similar to those found in normal animals.

Yellow phosphorus

Six rats were given one dose of 0·5 mg phosphorus. This large dose of phosphorus killed the animals in a few hours but no significant changes from normal values in the liver calcium or magnesium concentrations were observed. When rats were given 0·1 mg of phosphorus a day for 4 days, it was found that the liver calcium concentration had increased to about three times that of the normal, although the magnesium concentration remained unaltered.

Carbon tetrachloride

Three daily doses of carbon tetrachloride (0·05 ml in oil) increased the mean calcium concentration in the liver about seven times. The individual animal responded to a different extent to the toxin and hence the large standard error of the mean for this group. In an attempt to produce a more uniform response, four rats were given four times the original dose over the same period of time. In this experiment the calcium level was increased to about forty times the normal value but the variation was still large. In the same animals the magnesium concentration in the liver remained about the same as that for normal animals.

Thioacetamide

Gallagher *et al.*[1] first reported an increase in liver calcium after thioacetamide administration. We confirmed their results and found that one injection of 20 mg thioacetamide per 100 g body weight increased the liver calcium level approximately sixty-five times. However, the magnesium concentration in these livers was only about 50 per cent higher than the value for normal animals.

The toxins tried, regardless of the site of action caused an increase in calcium concentration when given in suitable doses.

The magnesium content of the liver is characterized by extreme stability when compared to the calcium content. After thioacetamide administration when the calcium concentration in the liver had increased over sixty times, the magnesium concentration only increased by about 50 per cent. For the other treatments no such increases in magnesium concentration were observed. It is unlikely that the bulk of calcium accumulating in necrotic tissue is in ionic form, but is most likely bound either to structural molecules or to a metabolic product such as citrate or inorganic phosphate. Gallagher *et al.*[1] found that the concentrations of citrate and calcium did not appear to be directly connected. The increases in total calcium may lead to an increase in ionic calcium which might then act as an ionic antagonist to magnesium in the activation of some cellular enzymes with resulting changes in the activity of some enzyme systems. For example, Lehninger[12], using a particulate fraction of rat liver, found that calcium ions were a potent inhibitor of oxidative phosphorylation.

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THE HYPERCALCEMIC ACTIVITY OF DIHYDROTACHYSTEROL$_2$ AND DIHYDROTACHYSTEROL$_3$ AND OF THE VITAMINS D$_2$ AND D$_3$ AFTER INTRAVENOUS INJECTION OF THE AQUEOUS PREPARATIONS—II

COMPARATIVE EXPERIMENTS ON RATS

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Abstract—In experiments on rats, the hypercalcemic activities of dihydrotachysterol$_2$, dihydrotachysterol$_3$, vitamin D$_2$ and vitamin D$_3$ have been compared at different time intervals (1, 2, 4 and 7 days) after the administration of one intravenous injection of the crystalline compounds dispersed in an aqueous medium.

It appears that dihydrotachysterol$_3$ is the most active hypercalcemic agent, followed, in decreasing order, by dihydrotachysterol$_2$, vitamin D$_3$ and vitamin D$_2$. This is exactly the same order that was found in a previous study of the effects of an oral administration of oily solutions of the same substances. The maximal serum calcium levels appear to be obtained 2–4 days after the intravenous injection.

The activity ratios prove to be highly dependent on the time interval again and show a fair agreement with the activity ratios found in the previous investigation.

INTRODUCTION

IN A previous report[3] the hypercalcemic activities of dihydrotachysterol$_2$, dihydrotachysterol$_3$, vitamins D$_2$ and D$_3$ have been compared at different time intervals after a single oral administration to rats of the crystalline compounds in pure peanut oil. Dihydrotachysterol$_3$ appeared to be the most active agent, followed in decreasing order by dihydrotachysterol$_2$, vitamin D$_3$ and vitamin D$_2$. The activity ratios proved to be highly dependent on the time interval, while the maximal serum calcium levels were reached 2 to 4 days after the administration.

Since the absorption by the intestinal wall played an important role in those experiments and this factor could have been determinant with regard to the ultimate effect in serum calcium increase, we decided to compare also the activity ratios of the four crystalline compounds at different time intervals after a single dose given by the parenteral route, notably the intravenous mode of administration.

EXPERIMENTAL

For intravenous injection, it was necessary to use aqueous dispersions of the substances under investigation. Therefore a 25%-aqueous solution of a solubilizer, belonging to the group of polyoxyethylene derivatives of fatty acid esters, was used for composing the preparations. For the determination of the hypercalcemic activity the

same method[1] was followed as used in our previous communication. For each series of experiments (dihydrotachysterol$_3$/dihydrotachysterol$_2$, dihydrotachysterol$_2$/vitamin D_3 or vitamin D_3/vitamin D_2), 180 male rats (average body weight $\pm$ 200 g) of our own inbred stock were prepared by feeding a stock diet, to which 1% calcium carbonate was added during at least 10 days. These animals were divided into five uniform groups four for the two substances to be compared (each compound at two dosage levels) and one control group.

On the day of dosing, all the rats received an intravenous injection of 0·25 ml of the respective aqueous preparations or the blank dilution medium (control group). The site of injection was the vena femoralis, made accessible by cutting off a little skin flap of the thigh (inner side). This manipulation as well as the injection was done under a slight either narcosis.

The quantities administered per rat (of about 200 g) were:

dihydrotachysterol$_2$	0·04 and 0·16 mg
dihydrotachysterol$_3$	0·01 and 0·04 mg
vitamin D_2	0·25 and 1·0 mg
vitamin D_3	0·25 and 1·0 mg

After 1, 2 and 4 days, respectively, twelve rats of each group were anaesthetized and their blood collected individually. The blood samples were centrifuged and the sera mixed in pairs (equal volumes) within the groups. In these mixed sera the calcium determinations were performed according to the method of Clark and Collip.[2]

With the choice of the parenteral route combined with the use of an aqueous dispersion, a rapid serum calcium increase could be expected and we thought the maximal time interval of 4 days would suffice at the onset.

Nevertheless an elongation of the time of observation appeared to be necessary in the course of the experiments, for which reason analogous experiments (60 rats) with a time interval of 1 week were added thereafter.

Each series of experiments was executed twice (experiments I and II), at different moments, several months apart. The obtained data were worked up statistically in pairs as in our foregoing study.

RESULTS AND DISCUSSION

In Tables 1, 2 and 3, the data of the series of experiments (1, 2 and 4 days) and of the separate experiments (1 week) are collected respectively for the following comparisons: dihydrotachysterol$_3$/dihydrotachysterol$_2$, dihydrotachysterol$_2$/vitamin D_3 and vitamin D_3/vitamin D_2. In all cases, the serum calcium values are expressed as increases over the corresponding control values (in mg %). Also the standard deviations of the mean values are given and, in parentheses, the numbers of the determinations made.

It is seen from the tables that maximal serum calcium increases occur at about 2–4 days after the intravenous injections. According to expectation, there is a clear trend of a somewhat more rapid serum calcium increase than in the previous experiments, when the compounds, dissolved in peanut oil, were administered orally.

On the basis of the data from the tables, the activity ratios have been calculated according to the method of *U.S. Pharmacopoeia*.[4] The results are summarized in Table 4. In the last column the weighted mean values of the duplicate determinations are given with their *P* 0·95 intervals.

TABLE 1. COMPARISON DIHYDROTACHYSTEROL$_3$ WITH DIHYDROTACHYSTEROL$_2$: MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg %)

Days after administering	Dihydrotachysterol$_3$		Dihydrotachysterol$_2$	
	0·01 mg	0·04 mg	0·04 mg	0·16 mg
1 (expt. I)	0·15 ± 0·20 (6)	0·48 ± 0·17 (6)	0·40 ± 0·12 (6)	1·27 ± 0·29 (6)
1 (expt. II)	0·38 ± 0·20 (6)	0·37 ± 0·13 (6)	0·11 ± 0·15 (6)	0·67 ± 0·15 (6)
2 (expt. I)	1·47 ± 0·13 (6)	2·72 ± 0·16 (6)	1·29 ± 0·14 (6)	2·74 ± 0·24 (6)
2 (expt. II)	1·08 ± 0·05 (6)	2·37 ± 0·08 (6)	1·07 ± 0·12 (6)	2·77 ± 0·33 (6)
4 (expt. I)	−0·16 ± 0·26 (6)	2·49 ± 0·29 (6)	0·15 ± 0·18 (6)	2·08 ± 0·09 (6)
4 (expt. II)	1·13 ± 0·10 (6)	2·93 ± 0·14 (6)	1·06 ± 0·13 (6)	2·74 ± 0·15 (6)
7 (expt. I)	0·48 ± 0·09 (6)	1·68 ± 0·12 (6)	−0·10 ± 0·10 (6)	0·81 ± 0·08 (6)
7 (expt. II)	−0·09 ± 0·06 (6)	1·07 ± 0·17 (6)	−0·15 ± 0·09 (6)	0·50 ± 0·03 (6)

TABLE 2. COMPARISON DIHYDROTACHYSTEROL$_2$ WITH VITAMIN D$_3$: MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg %)

Days after administering	Dihydrotachysterol$_2$		Vitamin D$_3$	
	0·04 mg	0·16 mg	0·25 mg	1·0 mg
1 (expt. I)	1·37 ± 0·13 (6)	1·88 ± 0·28 (6)	1·12 ± 0·19 (6)	1·20 ± 0·36 (6)
1 (expt. II)	0·47 ± 0·29 (6)	0·75 ± 0·21 (6)	0·56 ± 0·19 (6)	0·47 ± 0·08 (6)
2 (expt. I)	0·98 ± 0·19 (6)	3·44 ± 0·53 (6)	0·88 ± 0·19 (6)	3·56 ± 0·28 (6)
2 (expt. II)	1·08 ± 0·25 (6)	2·35 ± 0·28 (6)	1·39 ± 0·17 (6)	3·14 ± 0·48 (6)
4 (expt. I)	0·33 ± 0·11 (5)	1·46 ± 0·11 (6)	0·76 ± 0·11 (6)	2·47 ± 0·11 (6)
4 (expt. II)	0·07 ± 0·26 (6)	2·00 ± 0·19 (6)	0·55 ± 0·17 (7)*	2·14 ± 0·18 (6)
7 (expt. I)	0·08 ± 0·20 (5)	0·64 ± 0·11 (6)	0·18 ± 0·11 (6)	1·44 ± 0·31 (6)
7 (expt. II)	−0·02 ± 0·09 (6)	0·13 ± 0·17 (6)	0·02 ± 0·11 (6)	1·25 ± 0·30 (6)

* As two of the twelve serum samples have not been pooled, seven observations resulted.

TABLE 3. COMPARISON VITAMIN D$_3$ WITH VITAMIN D$_2$; MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg %)

Days after administering	Vitamin D$_3$		Vitamin D$_2$	
	0·25 mg	1·0 mg	0·25 mg	1·0 mg
1 (expt. I)	1·22 ± 0·14 (6)	1·13 ± 0·17 (6)	0·53 ± 0·10 (6)	0·94 ± 0·10 (6)
1 (expt. II)	0·35 ± 0·25 (6)	1·52 ± 0·20 (6)	−0·12 ± 0·09 (6)	0·65 ± 0·14 (6)
2 (expt. I)	1·46 ± 0·16 (6)	3·26 ± 0·27 (6)	1·07 ± 0·17 (6)	2·50 ± 0·41 (5)
2 (expt. II)	1·61 ± 0·11 (6)	3·19 ± 0·24 (6)	1·03 ± 0·09 (6)	3·19 ± 0·45 (6)
4 (expt. I)	0·95 ± 0·18 (6)	2·50 ± 0·24 (6)	0·60 ± 0·18 (6)	3·09 ± 0·21 (5)
4 (expt. II)	0·97 ± 0·04 (6)	2·58 ± 0·19 (6)	0·62 ± 0·11 (6)	2·15 ± 0·21 (6)
7 (expt. I)	−0·60 ± 0·09 (6)	0·61 ± 0·26 (6)	−0·89 ± 0·08 (6)	0·33 ± 0·13 (6)
7 (expt. II)	0·00 ± 0·09 (6)	2·09 ± 0·22 (6)	0·04 ± 0·07 (6)	1·79 ± 0·32 (6)

As can be seen from Tables 1, 2 and 3, the increases in serum calcium fail to show a sufficient dose–response relation in some cases, resulting in the absence of a reliable slope of the curve. The calculated activity ratios for those cases (1 and 7 days) are only approximate therefore and provided with a plus and minus sign in Table 4.

Table 4. Activity ratios at different times after administering

	Days after administ.	Expt. I	Expt. II	Weighted mean value and P 0·95 interval
Dihydrotach. 3/ Dihydrotach. 2	1	± 1·2	± 3·6	± 2·4 —
	2	4·35	3·36	3·80 77–130%
	4	4·12	4·43	4·31 84–119%
	7	10·40	± 6·50	± 8·40 —
Dihydrotach. 2/ vit. D_3	1	± 56	± 24	± 40 —
	2	6·2	3·8	5·6 71–142%
	4	± 3·1*	4·9	± 3·8 (63–159)%
	7	± 3·1	± 1·9	± 2·4 —
Vit. D_3/vit. D_2	1	± 11·0	2·60	± 6·8 —
	2	1·62	1·43	1·50 71–140%
	4	0·93	1·41	1·12 79–126%
	7	1·38	1·10	1·20 78–128%

* The two lines fail to be statistically parallel.

The hypercalcemic activity of vitamin D_2 appears to be the weakest. It is followed, in increasing order, by vitamin D_3, dihydrotachysterol$_2$ and dihydrotachysterol$_3$, which is exactly the same order as was found in our previous experiments with oral application of the oily solutions.

The mutual activity ratios find expression in Table 5, in which we have put the hypercalcemic activity of dihydrotachysterol$_2$ equal to one for each time interval (2, 4 and 7 days), and recalculated the activity relations. For convenience we repeat in this table the data of our previous study ("oily oral").

Table 5. Activity ratios (dihydrotachysterol$_2$ equal to one)

Application:	"Aqueous intravenous"			"Oily oral"		
Days after dosing:	2	4	7	2	4	7
vitamin D_2	0·12	0·23	± 0·35	0·15	0·20	0·20
vitamin D_3	0·18	0·26	± 0·42	0·24	0·39	0·28
dihydrotachysterol$_2$	1·0	1·0	1·0	1·0	1·0	1·0
dihydrotachysterol$_3$	3·8	4·3	± 8·4	3·6	6·0	3·4

In general a fair agreement seems to exist between the two series of values.

As we now dispose of the results of the dihydrotachysterols and of the vitamins D in different media and with different modes of application, viz. "oily oral" and "aqueous intravenous", it appears interesting to demonstrate the influence thereof.

This can be done by calculating the average serum Calcium increase for each compound and at each level from this paper and from our previous publication from all the experiments given in the Tables 1, 2, 3 and 1a, 1b, 1c, respectively. The results

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are given in Tables 6, 7, 8 and 9. They clearly show a better utilization of the four compounds on a weight basis when intravenously given as aqueous dispersions. These obvious differences will be caused by losses and a limitation of the rate of absorption

TABLE 6. COMPARISON VITAMIN D_2 "OILY ORAL" WITH "AQUEOUS INTRAVENOUS"; MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg %)

Days after dosing	"Oily oral"			"Aqueous intravenous"	
	1·0 mg	4·0 mg	16·0 mg	0·25 mg	1·0 mg
1	0·34	1·12	0·80	0·20	0·80
2	0·95	2·71	4·64	1·05	2·88
4	0·52	2·61	4·14	0·61	2·58
7	−0·09	1·57	2·79	−0·42	1·06

TABLE 7. COMPARISON VITAMIN D_3 "OILY ORAL" WITH "AQUEOUS INTRAVENOUS"; MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg %)

Days after dosing	"Oily oral"				"Aqueous intravenous"	
	0·25 mg	1·0 mg	4·0 mg	16·0 mg	0·25 mg	1·0 mg
1	—	0·90	0·80	0·90	0·81	1·08
2	0·79	1·48	3·54	4·79	1·34	3·29
4	0·60	1·57	3·52	5·29	0·80	2·42
7	0·49	0·81	2·38	3·16	−0·10	1·35

TABLE 8. COMPARISON DIHYDROTACHYSTEROL$_2$ "OILY ORAL" WITH "AQUEOUS INTRAVENOUS"; MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg %)

Days after dosing	"Oily oral"			"Aqueous intravenous"	
	0·25 mg	1·0 mg	4·0 mg	0·04 mg	0·16 mg
1	0·88	1·36	1·64	0·59	1·14
2	1·66	3·56	4·70	1·10	2·82
4	1·63	3·61	4·47	0·41	2·07
7	0·96	2·49	3·67	−0·05	0·52

TABLE 9. COMPARISON DIHYDROTACHYSTEROL$_3$ "OILY ORAL" WITH "AQUEOUS INTRAVENOUS"; MEAN INCREASE SERUM CALCIUM OVER CONTROLS (mg %)

Days after dosing	"Oily oral"			"Aqueous intravenous"	
	0·1 mg	0·4 mg	1·6 mg	0·01 mg	0·04 mg
1	1·11	1·25	1·06	0·26	0·42
2	2·33	3·74	5·50	1·28	2·54
4	3·22	4·58	5·46	0·48	2·71
7	1·20	2·81	4·07	0·20	1·38

through the intestinal wall in the case of oral administration of the solution in oil. These factors are probably of the same order of magnitude in view of the fair similarity, which appears to exist between the activity ratios of the four compounds investigated by two different modes of administration (see Table 5).

A quantitative evaluation of the data of the Tables 6–9 is not justified on statistical grounds, but two days after the administration an activity ratio: "aqueous intravenous"/"oily oral" in the order of 3 to 4 could be estimated for each of the four compounds.

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SOME EFFECTS OF D_2O *IN VIVO* AND *IN VITRO* ON CERTAIN ENZYMES OF RAT TISSUES*

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Abstract—In animals that had drunk 50% heavy water until about one-third of their body water was replaced by D_2O, changes were observed in the tissue levels of several enzymes. The arginase and oxalacetate transaminase activities of rat liver were significantly increased; alanine transaminase was unchanged. Tryptophan pyrrolase was elevated slightly in livers of unadapted rats; however, there was marked suppression of the increase in activity induced by injection of tryptophan. The activity of glutamic acid decarboxylase of rat brain was depressed by D_2O *in vitro*, but the concentration of this enzyme was not changed by feeding D_2O. Mitochondria prepared from liver and kidney of D_2O-treated rats showed a diminished capacity to oxidize fumarate and α-ketoglutarate, but not succinate; the efficiency of phosphate esterification was unaffected. D_2O (30%) *in vitro* did not affect transaminase activity or oxidative phosphorylation.

ADMINISTRATION of D_2O to an animal represents a situation which is unique in toxicology, since profound changes can be produced in an organism merely by alteration of the relative abundance of different stable isotopes of the same element. The effects of feeding D_2O to mammals were first studied systematically by Barbour[1] and co-workers. However, the types of experiments they could do were limited by the scarcity of heavy water. In recent years, with more adequate supplies of D_2O available, the mechanism of toxic action of D_2O has been reinvestigated.[2] One approach has been to assay tissues of D_2O-treated animals for various enzymes. Preliminary studies showed that the catalase, esterase, and DPN-cytochrome *c* reductase activities of rat liver decreased to about two-thirds of the normal value in animals in which 32 per cent of the body water had been replaced by D_2O. The levels of arginase and uricase were increased, while those of cytochrome oxidase and succinic dehydrogenase were decreased slightly.[2] This report is concerned with assays of some other enzymes in rat tissues.

METHODS

Sprague–Dawley rats, from 4 to 7 months old, were used in these experiments. For the study of the effects of D_2O *in vivo*, they were given 50% D_2O as their drinking water, and were fed Rockland rat diet *ad libitum*. Urine samples were collected periodically and assayed for D_2O to provide an index of replacement of body water by D_2O. Immediately before the rats were killed, a sample of blood was taken by heart puncture, and the D_2O content of the plasma was measured spectrophotometrically.[2] The effects of D_2O *in vitro* were studied on tissues of normal rats.

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Rats were killed by decapitation. The organs were rapidly removed, chilled in cracked ice, then homogenized by means of a Potter–Elvehjem homogenizer in a suspending medium appropriate for the assay of the enzyme under investigation. In different experiments, tissues were assayed for arginase,[3] transaminase,[4] tryptophan pyrrolase,[5] glutamic acid decarboxylase[6] and oxidative phosphorylation.[7] In the last-named assay, mitochondria were prepared from rat liver by gradient centrifugation.[8] Tryptophan pyrrolase was measured in livers both of unadapted rats and of rats injected intraperitoneally 6 hr before they were killed, with 2 g of DL-tryptophan per kg of body weight.

RESULTS

Arginase

Preliminary data on arginase activity in livers of D_2O-treated rats had shown an increase of 18 per cent in rats with about one-fifth of their body water replaced by D_2O.[2] This figure was based on data obtained from only three treated rats and two controls, so that confirmation was necessary. Table 1 shows the results of an experiment which began with six rats in each group; one of the D_2O-treated animals died

TABLE 1. ARGINASE ACTIVITY IN LIVERS OF D_2O-TREATED RATS

	Control	D_2O-treated
Number of rats	6	5
Days on 50% D_2O	—	32
Final plasma D_2O (%)	—	31·5
Range		(31·1–31·7)
Weight change (g)	+27	−9
Range	(+25 to +30)	(−24 to +12)
Arginase activity*	461 ± 21	528 ± 50

* μmoles arginine destroyed per 10 min per mg N, averages and standard deviation.

on the twenty-ninth day, with a plasma D_2O level of 30 per cent. The arginase activity of the livers of the treated rats was about 15 per cent higher than that of the control livers; this difference is statistically significant ($P < 0{\cdot}05$).

Transaminase

Two transaminase reactions were measured in liver homogenates: the formation of oxalacetate from α-ketoglutarate and aspartate, and the formation of alanine from pyruvate and glutamate. *In vitro*, neither was affected by 30% D_2O. Alanine transaminase was inhibited about 15 per cent in 50% D_2O; in three experiments, the inhibitions observed were 12, 16, and 17 per cent. Oxalacetate transaminase was not studied at the 50 per cent level *in vitro*. The *in vivo* changes are shown in Table 2. No effect was observed with alanine transaminase, while the activity of oxalacetate transaminase was increased by nearly 20 per cent ($P = 0{\cdot}05$). In this experiment, the blood glucose and urea were also measured; the level of the former was reduced to about three-fourths of normal, while the latter was more than double the normal value.

Tryptophan pyrrolase

This enzyme was of particular interest since its level in rat liver can vary in different physiological states. Fourteen rats were given 50% D_2O to drink; one rat died after

TABLE 2. TRANSAMINASE ACTIVITY IN LIVERS OF D_2O-TREATED RATS

	Control	D_2O-treated
Number of rats	9	7
Days on 50% D_2O	—	26
Final plasma D_2O (%)	—	31·7
Range		(29·5–34·0)
Weight change (g)	+42	+6
Range	(+22 to +61)	(−13 to +28)
Blood glucose (mg %)	80	62
Range	(61–91)	(45–83)
Blood urea (mg %)	18·9	44·3
Range	(13·8–21·4)	(28·7–58·0)
Transaminase*		
Alanine	24·9 ± 1·1	22·5 ± 1·8
Oxalacetate	67·8 ± 9·9	80·4 ± 9·6

* μmoles product formed per hr per g N, averages and standard deviations.

28 days. An increase in activity was observed in livers of rats killed after 11 days, and after 32 days the tryptophan pyrrolase activity was almost 40 per cent above normal (Table 3). Only four controls were used in this experiment; however, since the values observed in these rats corresponded well with those obtained on fifty-four rats used in other experiments, additional controls were considered unnecessary.

TABLE 3. TRYPTOPHAN PYRROLASE ACTIVITY IN UNADAPTED RATS TREATED WITH D_2O

Days on D_2O	Plasma D_2O, ave. % and range	Ave. weight loss (g)	No. rats	μmoles kynurenine formed per hr/g dry wt.		
				Ave.	σ	*P*-value
0	—	—	4	7·4	0·6	—
			54*	7·7	1·5	—
11	13·6 (13·0–14·2)	4	4	8·8	0·5	0·05
21	26·8 (24·3–27·8)	24	4	8·3	0·9	0·2
32	30·3 (29·2–32·9)	36	5	10·2	1·8	0·03

* Control data from earlier experiments.[8]

The adaptive response to intraperitoneal injection of 2 g of DL-tryptophan per kg was also studied in D_2O-treated animals. Two series of experiments were carried out. In the first experiment, animals were killed after 17, 24 and 31 days; since the values for plasma D_2O were very close for the first two groups, the data for 17 and 24 days have been pooled. For the same reason, the results of the second experiment, in which the rats were killed on the seventh and twelfth days, have been similarly treated.

The rats used in this second experiment were younger and lighter than those used in the first experiment, and accumulated D_2O more rapidly.

It is clear that deuteration markedly reduced the capacity of the rat to respond to the injection of tryptophan (Table 4). Instead of the usual twelve- to fifteen-fold increase in activity seen in normal rats, the activity was increased less than three-fold in rats with 30 per cent replacement. This change is detectable even at 18 per cent

TABLE 4. EFFECT OF FEEDING D_2O ON INDUCTION OF TRYPTOPHAN PYRROLASE IN RAT LIVER BY INTRAPERITONEAL INJECTION OF TRYPTOPHAN

Days on D_2O	Plasma D_2O, ave., % and range	Ave. weight loss (g)	No. rats	μmoles kynurenine formed per hr/g dry wt.	
				Ave.	σ
0	—	—	4	101	16
			12*	121	18
17–24	26·7 (23·5–28·8)	31	6	41	14
31	34·6 (34·0–36·2)	53	4	36	7
0	—	—	4	100	15
7–12	18·1 (15·9–19·8)	1	7	80	19

* Control data from earlier experiments.[9]

replacement, when the animals are essentially free of signs of toxicity, and becomes very marked in moribund animals. One rat among those injected with tryptophan on the thirty-first day failed to survive the 6-hr period after injection.

Glutamic acid decarboxylase

In vitro, 30% D_2O effected a reduction of glutamic acid decarboxylase activity of rat brain to about 85 per cent of normal. The individual values of three experiments were 88, 87 and 78 per cent. However, the glutamic acid decarboxylase activity of brains of D_2O-treated rats did not differ significantly from the controls. The average value for seven treated rats, with an average plasma D_2O of 29·2 per cent, was 45·0 μl CO_2 formed per hr per mg N, while for the controls it was 46·8. The assays were carried out in ordinary water.

Oxidative phosphorylation

Mitochondria isolated from liver and kidney of rats drinking D_2O were tested for their capacity to esterify inorganic phosphate. Three different substrates, succinate, fumarate and α-ketoglutarate, were used in these experiments. Four rats were designated for the experiment, but one died before it could be used. The average value for plasma D_2O of the other three was 32·0 per cent. Although the numbers of animals employed were small, the results seem clear-cut (Table 5).

In no case was the efficiency of phosphorylation impaired; the P/O ratios for all three substrates with both liver and kidney mitochondria were normal. However, the rates both of oxygen consumption and of phosphate esterification were slowed from 15 to 25 per cent with either fumarate or α-ketoglutarate as substrates. The inhibition was considerably less with succinate.

In vitro, with fumarate as the substrate, 30% D_2O had little effect on the reaction. At 60% D_2O, oxygen consumption was decreased by 25 per cent and phosphate esterification by 30 per cent.

DISCUSSION

One of the features of D_2O intoxication in rats is the evident involvement of the adrenals. There is an adrenal hypertrophy, the weight of the glands nearly doubling

TABLE 5. OXIDATIVE PHOSPHORYLATION IN LIVER AND KIDNEY MITOCHONDRIA OF NORMAL AND D_2O-TREATED RATS

	Substrate					
	Succinate	Fumarate	α-Keto-glutarate	Succinate	Fumarate	α-Keto-glutarate
	Normal liver			*Normal kidney*		
No. rats	12	25	7	3	3	5
μ moles P esterified/ 20 min per mg N	19·5 ± 3·0	19·2 ± 3·1	19·2 ± 2·8	46·1 (42·6–50·8)	45·5 (40·4–52·0)	43·0 (41·0–49·2)
μ atoms O consumed/ 20 min per mg N	10·5 ± 1·7	6·8 ± 0·9	5·2 ± 0·8	26·2 (26·0–26·4)	15·9 (15·0–16·3)	12·6 (12·2–12·8)
P/O ratio	1·9 (1·5–2·0)	2·8 (2·6–3·0)	3·6 (3·4–4·1)	1·9 (1·8–2·0)	2·9 (2·9–3·0)	3·6 (3·2–4·0)
	D_2O *liver*			D_2O *kidney*		
No. rats	3	3	3	3	3	3
μ moles P esterified/ 20 min per mg N	17·8 (17·0–18·8)	14·4 (13·0–16·6)	15·9 (15·0–16·5)	47·4 (37·6–50·2)	33·8 (24·9–39·6)	35·7 (28·4–39·9)
μ atoms O consumed/ 20 min per mg N	9·0 (8·1–10·1)	5·1 (4·9–5·4)	4·4 (4·3–4·7)	25·8 (24·1–28·2)	11·9 (9·9–13·6)	11·0 (8·8–12·9)
P/O fatio	2·0 (1·9–2·1)	2·8 (2·6–3·1)	3·5 (3·1–3·9)	1·8 (1·5–2·1)	2·8 (2·6–3·0)	3·2 (3·1–3·4)

Data are expressed as averages with ranges or averages ± standard deviations

at one-third replacement of body water by D_2O.[2] Hypophysectomized rats are much more susceptible to the lethal action of D_2O than are unoperated controls, presumably because of their impaired capacity to adapt to the altered internal environment. Kidney function is depressed,[10] but since the capacity of slices of kidney from D_2O-treated rats to esterify *p*-aminohippuric acid (PAH) *in vitro* is unimpaired,[11] the depression of PAH clearance in the intact animal may be attributed to disturbed adrenal function. The blood picture in moribund D_2O-treated rats resembles that seen in adrenalectomized animals, with high NPN, urea, and lactic acid, and low glucose and plasma protein.

It has been demonstrated that the arginase and transaminase activities of rat liver decrease after adrenalectomy.[9, 12] Since a decrease was not seen in these experiments, the concept that the deuterated rat behaves as if it had been adrenalectomized is untenable, even though some functions of the adrenal are apparently suppressed; the production of adrenal hormones does not seem to be inhibited. It is possible that the rat treated with D_2O may have a higher requirement for adrenal hormones than could be met even by a hypertrophic adrenal cortex, or that the metabolic processes of the rat are so affected by deuteration that no additional amount of adrenal hormones could benefit the animal.

The increase in activity of tryptophan pyrrolase in D_2O-treated rats is consistent with the idea that adrenal activity is unimpaired or even somewhat increased, since the level of this enzyme can be raised by administration of hydrocortisone and to a lesser extent cortisone.[13] However, it is also possible that this increase in activity may result from an increased concentration in the body of products of protein breakdown, since a number of amino acids and their derivatives are known to produce an elevation of tryptophan pyrrolase in the normal rat.[5, 14]

It is particularly interesting to note that the adaptive response of this enzyme to the injection of tryptophan was strongly inhibited. Although the data of Gros *et al.*[15]

indicate that the increase of tryptophan pyrrolase produced by administration of its substrate involves actual synthesis of additional protein, some recent work[16, 17] indicates that *de novo* synthesis may not be the complete explanation for the tryptophan-induced augmentation of tryptophan pyrrolase. Hence, it is difficult to state that these data demonstrate an inhibition of protein synthesis, although such an interference would not be inconsistent with other effects of D_2O.

Although the concentration of glutamic acid decarboxylase was not measurably decreased in the brains of rats D_2O, 15 per cent inhibition was produced *in vitro* by D_2O. Thus, while the level of the enzyme was not affected, its physiological activity *in vivo* could be diminished by replacement of tissue fluids by heavy water. Such an inhibition might explain some of the effects of D_2O on the central nervous system, since a decreased level of γ-aminobutyric acid is associated with hyperexcitability.[18]

The data on oxidative phosphorylation by mitochondria prepared from tissues of D_2O-treated rats showed that with the substrates which are dehydrogenated via DPN, i.e. fumarate and α-ketoglutarate, there was an appreciable reduction in oxidative activity that was not seen with succinate as substrate. Earlier data had demonstrated a decrease in DPN–cytochrome *c* reductase in liver; the levels of this enzyme in kidney were not measured in rats with plasma D_2O values above 20 per cent. Conceivably a deficiency of this enzyme could account for the inhibition of oxidative phosphorylation, although it has not been established whether DPN–cytochrome *c* reductase is lost from mitochondria, microsomes, or both. Furthermore, the significance of the decrease is questionable, since there is no indication of the importance of the influence of D_2O on these reactions in the intact animal. Whether a 35 per cent decrease in DPN–cytochrome *c* reductase would disturb the economy of the rat is not known. Since the size of the liver is increased by as much as 50 per cent in heavily-deuterated rats,[2] the decrease of an enzyme to two-thirds of the normal level would still entail no loss in *total* organ activity.

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INHIBITION OF ELECTRON TRANSPORT BY ANTIMYCIN A, ALKYL HYDROXY NAPTHOQUINONES AND METAL COORDINATION COMPOUNDS

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Abstract—Inhibition studies of electron transport by antimycin A and its derivatives demonstrated the requirement for available ligand groups and the lipophilic part of the molecule. Inhibition may be caused by the co-ordination of ion in the lipid molecular environment of the electron transport chain. Alkyl hydroxy naphthoquinones may inhibit by a similar mechanism or they may compete with coenzyme Q as their inhibition of succinic dehydrogenase-coenzyme Q indicates. Some conventional metal co-ordination compounds, especially thenoyl trifluoroacetone, are good inhibitors of electron transport but they lack specificity.

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INTRODUCTION

THE antibiotic, antimycin A,[1] the dihydrostreptomycin antagonist, 2-heptyl-4-hydroxyquinoline N-oxide,[2–4] and some of the naphthoquinone antimalarials,[5–8] are potent inhibitors of electron transport. Inhibition of the succinate and the reduced diphosphopyridine nucleotide (DPNH) chains by any of these three types of compounds blocks the cytochrome *b* in the reduced state and cytochrome c_1 in the oxidized state.[3–9] This region of electron transport has recently been characterized for the succinate chain as:

succinic dehydrogenase → cytochrome *b* → coenzyme Q—cytochrome c_1[10]

This part of the chain is known to contain two lipoproteins and non-heme iron.[11, 12] The site of antimycin A inhibition is between coenzyme Q and cytochrome c_1[13] but the site for the other two inhibitors has not been fully localized. Since these three inhibitors have two structural similarities in common, namely, ligand groups and C_6 to C_9 alkyl side chains, the mechanism of inhibition may involve co-ordination of non-heme iron in a lipid molecular environment. It is the purpose of this paper to report studies of the mechanism of inhibition by antimycin A, by alkyl hydroxy naphthoquinones and by some metal co-ordination compounds.

MATERIALS AND METHODS

Antimycin A and its derivatives were kindly supplied by Prof. F. M. Strong, University of Wisconsin. The 3-alkyl derivatives of 2-hydroxy-1, 4-naphthoquinone were supplied by Prof. L. F. Fieser, Harvard University.

Succinic- and DPNH-cytochrome *c* reductase activities were measured by recording the increasing absorbance at 550 mμ of cytochrome *c* reduction as a function of time using a Bechman DK spectrophotometer. Percentage inhibition by animtycin A and derivatives and by the metal co-ordination compounds was calculated from these recorded reaction rates. The reaction system contained 5×10^{-3} M succinate or DPNH, 1×10^{-3} M cyanide, 1 mg of cytochrome *c* per ml of electron transport particle preparation[14] containing 0·1 mg protein, and 0·1 M phosphate buffer, pH 7·5. Inhibitors were added to the reaction system in small amounts of ethanol, the reaction system was brought to the reaction temperature of 37 °C in 2 min, and the electron transport preparation was added to start the reaction.

A preparation of purified succinic dehydrogenase–coenzyme Q was supplied by D. M. Ziegler. Succinic dehydrogenase–coenzyme Q activity was measured at 37 °C by recording decreasing absorbance at 600 mμ of dichlorophenolindophenol reduction as a function of time. The reaction system contained 4×10^{-3} M succinate, 1×10^{-4} M Versene, 3×10^{-5} M 2 : 6-dichlorophenolindophenol, 5×10^{-2} M phosphate buffer, pH 7·4, and 3 μg (protein) succinic dehydrogenase-coenzyme *Q* preparation. Naphthoquinone and metal co-ordination inhibitors were added either before or during the reaction.

RESULTS AND DISCUSSION

Ligand groups of the known inhibitors

Although the structure of antimycin A (Ia + Ib) has not been established with certainty,[1] the blastomycic acid structure (Ia) is sufficiently known to locate a number of ligand groups which may form chelate rings with iron or other metals. Firstly, the 1-carbonyl group together with the 2-hydroxyl group are a pair of ligands which should have co-ordination properties similar to other analogs of salicyaldehyde or salicylic acid. Secondly, the 2-hydroxyl together with the secondary 3-amide are a pair of ligands somewhat similar to aminophenols. Thirdly, the carboxyl group with its α-secondary amine are a ligand pair similar to amino acids. On the basis of available stability constants,[15–17] the 1-carbonyl-2-hydroxyl groups should be the strongest ligands. Further, the stability of a didentate six-membered metal chelate formed with the 1-carbonyl-2-hydroxyl groups should be increased by the proximity of the other ligand groups. A study of the stability constants of the ligand groups of antimycin A will be very useful, but this must await the completion of structural studies. Antimycin A is known to form a blue-colored co-ordination compound with ferric chloride.

That the 2-hydroxy-3-alkyl naphthoquinones (II) should co-ordinate metals and form chelates may be inferred from information concerning structural analogs. Metal chelates of *o*-hydroxyquinone and 1-hydroxyanthroquinone have been studied in detail.[15, 17] Many dyes containing the *o*-hydroxyquinoid structure, particularly alizarin dyes, are known to co-ordinate iron and other metals.

2-Heptyl-4-hydroxyquinoline-N-oxide (III) has two ligand groups, the hydroxyl and the quinoline nitrogen, which may co-ordinate metals. A co-ordination compound which is colored orange-red is formed when 2-alkyl-4-hydroxyquinoline N-oxides are reacted with ferric chloride.[2]

Inhibition of succinate–cytochrome c *reductase activity by antimycin A and its derivatives*

To define the structural requirements of antimycin A as an inhibitor, all currently-

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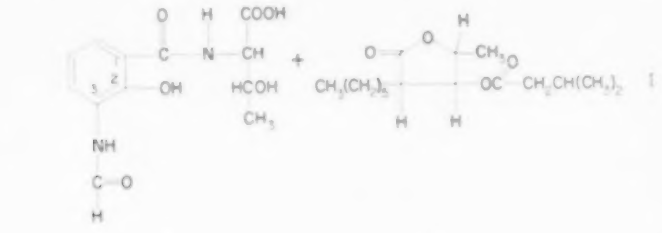
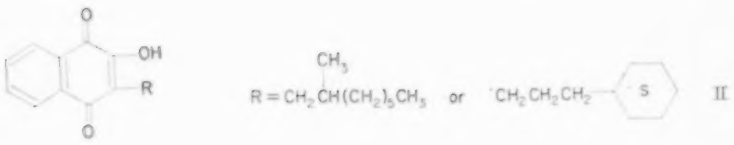
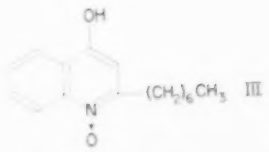
available derivatives were tested, and the results are shown in Table 1. The succinate–cytochrome *c* reductase reaction was chosen as the simplest for evaluating inhibitors that inhibit between cytochromes *b* and c_1. The three fractions of antimycin, A_1, A_2 and A_3, gave 50 per cent inhibition between 0·01 and 0·05 μg per ml. Thus, these three



a b



II



III

chromatographic fractions, which have very similar physical and chemical properties,[1] have similar biological activity. When the formyl group on the 3-amine is removed, as in deformylated antimycin A_1 hydrochloride, the inhibitory power drops by a factor of 10. Structural considerations given here implicate the phenolic 2-hydroxyl as a metal co-ordinating ligand group. The decreases in inhibition, when this acidic hydroxyl group is substituted, as in antimycin A_3-monomethyl ether and diacetyl-blastomycin, are most interesting. Conversion of the hydroxyl to the monomethyl ether has a profound effect. The concentration of the ether must be a thousand times greater than antimycin A_3 for equal inhibition. Diacetylblastomycin is one-tenth as inhibitory as blastomycin.

Since both blastomycic acid (Ia) and the structurally similar antimycic acid contain the ligand groups but lack the neutral lipid-soluble gragment (Ib) of antimycin, their lack of strong inhibitory power is an indication of the essentiality of the lipophilic part of antimycin. Blastomycic acid was found to be less than one-thousandth as inhibitory as antimycin. It has an inhibitory power similar to that of the classical metal co-ordination compounds which will be described later in this study. Antimycic acid could not be tested at higher concentrations because it reduced cytochrome *c* directly and thus interfered with the enzymic measurement. The available derivative closest to the neutral fragment (Ib) is 1-methyl-2-hydroxyl-3-*n*-hexyl butyrolactone which was less than one-thousandth as inhibitory as antimycin.

Inhibition of succinic dehydrogenase–coenzyme Q activity by alkyl hydroxy naphthoquinones

Purified succinic dehydrogenase–coenzyme Q was chosen to determine if it contained a site of inhibitions. Poor inhibition was obtained with 2-heptyl-4-hydroxyquinoline N-oxide with 19 per cent inhibition at 5 μg per ml, 57 per cent at 14 μg per ml, and 81 per cent at 35 μg per ml. Since inhibition of overall electron transport does not require such large amounts, we can conclude that the succinic dehydrogenase–coenzyme Q preparation does not contain its site of inhibition. In contrast, 3-alkyl-2-hydroxyl-1 : 4-naphthoquinones proved to be good inhibitors; inhibition by a number

TABLE 1. INHIBITION OF SUCCINATE-CYTOCHROME *c* REDUCTASE ACTIVITY BY ANTIMYCIN A AND DERIVATIVES

Inhibitor	Concentration (μg/ml)	Inhibition (%)
Antimycin A_1	0·01	5
	0·25	89
	0·25	94
	0·5	100
Antimycin $A_{2'}$	0·01	47
	0·05	97
Antimycin A_3 (Blastomycin)	0·01	40
	0·05	96
Deformylated antimycin A_1 hydrochloride	0·01	0
	0·05	18
	0·5	91
Antimycin A_3-monomethylether	0·5	0
	5·0	42
	50·0	94
Diacetylblastomycin	0·05	0
	0·25	50
	0·5	90
	5·0	100
Antimycic acid	5·0	20
Blastomycic acid	0·5	0
	5·0	18
	50·0	34
1-Methyl-2-hydroxyl-3-*n*-hexyl butyrolacetone	5·0	0
	50·0	23

Reaction system contained 5×10^{-3} M succinate, 1×10^{-3} M cyanide, 1 mg per ml. Cytochrome *c*, electron transport particle preparation containing 0·1 mg protein, and 0·1 M phosphate buffer, pH 7·5. Percentage inhibition was calculated from reaction rates of cytochrome *c* reduction.

of alkyl derivatives is given in Table 2. Some of these are better inhibitors than SN5949, which has been mainly used[6–8] in research on the electron transport chain. Among the naphthoquinone derivatives tested, the inhibitory power increases with increases in the lipophilic character of the side chain. Polar hydroxyl or carbonyl groups in the middle of long lipophilic side chains do not decrease the comparative

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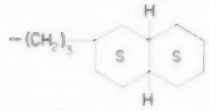
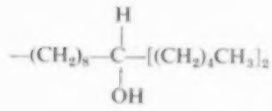
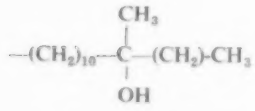
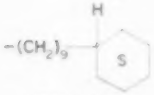
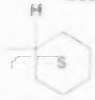
inhibitory power, but the carboxyl group on the C- alkyl does. Among inhibitors of the electron transport system these are the most inhibitory quinones known.

TABLE 2. INHIBITION OF SUCCINIC DEHYDROGENASE-CoQ ACTIVITY BY 3-ALKYL DERIVATIVES OF 2-HYDROXY-1 : 4-NAPHTHOQUINONE

3-Alkyl side chain	Concentration (μg/ml)	Inhibition (%)
	0·5 1 2	85 85 94
$-(CH_2)_{12}-CH_3$	0·5 1 2	90 90 90
	1 4	85 100
	1 4	78 100
$-(CH_2)_7-C(=O)-(CH_2)_7CH_3$	1	73
	1 4	71 94
$-(CH_2)_9CH_3$	1 4	63 86
$-CH_2CH(CH_3)(CH_2)_5CH_3$ (SN 5949)	1 4	48 92
	1 4	38 39
$-(CH_2)_9COOH$	1 4 16	29 37 68

Reaction system contained 4×10^{-3} M succinate, 1×10^{-4} Versene, 3×10^{-2} M 2 : 6-dichlorophenolindophenol, 5×10^{-2} M phosphate buffer, pH 7·4 and succinic dehydrogenase-coenzyme Q preparation containing 3 μg protein. Percentage inhibition was calculated from reaction rates of indophenol reduction.

Because these naphthoquinones may inhibit at coenzyme Q_{10}, 3-$(CH_2)_3$-decalyl-2-hydroxy-1 : 4-naphthoquinone was tested for competitive inhibition. The results in Table 3 show that it was competitive with externally added coenzyme Q_{10} and also, coenzyme Q_7, coenzyme Q_2 and vitamin K_1. Competition with coenzyme Q_{10} for

reaction with metals may be the mechanism by which these naphthoquinones inhibit the electron transport system. However, this mechanism cannot be definitely established with this system because the competitive effect of coenzyme Q_7, coenzyme Q_2 and vitamin K_1 shows that the competition is not specific.

The fact that vitamin K_1 overcomes the inhibition can be considered as evidence of non-specificity because vitamin K_1, will not replace coenzyme Q_{10} in extracted mitochondria,[18] electron transport particle[19] and cytochrome *c* reductase.[20] Coenzyme Q_{10} and its closely related homologs show good specificity in replacing the coenzyme Q_{10} extracted from these particles. In order to localize the site and to determine the

TABLE 3. COMPETITION OF QUINONES WITH NAPHTHOQUINONE INHIBITOR

Concentration of 3-$(CH_2)_2$-decalyl-2-hydroxy-1 : 4-naphthoquinone (μg/ml)	Quinone	Concentration of quinone (μg/ml)	Inhibition of indophenol reaction (%)
1	CoQ_{10}	0	79
1		4	52
1		8	16
4		8	79
4		28	71
1	CoQ_7	0	77
1		2	61
1		5	50
2		5	67
5		5	81
1	CoQ_2	0	100
1		0·8	0
9		0·8	37
1	Vitamin K_1	0	84
1		1	50
6		2	100
6		10	92

Reaction system and methods are as described for Table 2.

mechanism of alkyl hydroxy naphthoquinone inhibition it will be necessary to study the internal coenzyme Q_{10} of intact mitochondria, as was done with antimycin A inhibition.[13]

When coenzyme Q_{10} and coenzyme Q_2 were added alone to the succinic dehydrogenase–coenzyme Q system, the externally added coenzyme Q_{10}, up to 20 μg per ml, did not increase activity, whereas coenzyme Q_2, at 0·8 μg per ml, increased the rate by 50 per cent. For each naphthoquinone tested and listed in Table 2, 0·8 μg of coenzyme Q_2 per ml overcame the inhibition of 1 μg of naphthoquinone per ml.

Inhibition of electron transport by metal co-ordination compounds

Another approach to defining the physical and chemical requirements for inhibitors of the succinate to cytochrome *c* part of the electron transport chain is to study well-known metal co-ordination compounds which are lipophilic. Eighteen co-ordination compounds were selected[15, 17, 21] and tested in attempts to find powerful inhibitors. Compounds for which complete inhibition data were obtained are compared in Table 4.

The following co-ordination compounds gave essentially no inhibition up to the concentration given: 8×10^{-3} M acetyl acetone, 1×10^{-2} M benzoylacetone, 2×10^{-3} M 8-hydroxyquinoline, 1×10^{-3} M cupferron, 5×10^{-3} M dimethylglyoxine, 3×10^{-3} M dihydroxyethylglycine, 1×10^{-3} M phenylalanine, and 1×10^{-3} M *iso*octylthioglycolate. This showed that these compounds are not strong inhibitors, and they were not tested further. All of the inhibitory compounds (Table 4), except mercaptosuccinic acid, are known to form metal inner complexes which are soluble in lipid solvents,[15, 17] Mercaptosuccinic acid is similar to carboxymethylmercaptosuccinic acid, which is known to chelate iron and copper in lipid solution.[22] The best inhibitors found, 5×10^{-5} M 2-thenoyl trifluoroacetone and 5×10^{-5} M 4 : 7-diphenyl-2 : 9-dimethyl-1 : 10-phenanthroline, can be compared to the three

TABLE 4. INHIBITION OF ELECTRON TRANSPORT SYSTEMS BY METAL CO-ORDINATION COMPOUNDS

Co-ordination compound	Concentration for 50% inhibition ($M \times 10^5$)		
	Succinate–cytochrome *c* reductase	DPNH–cytochrome *c* reductase	Succinic dehydrogenase–coenzyme Q
2-Thenoyltrifluoroacetone	5	200	1
2-Furoyltrifluoroacetone	200	—	10
4 : 7-Diphenyl-2 : 9-dimethyl-1 : 10-phenanthroline	5	20	1
4 : 7Diphenyl-1 : 10-phenanthroline	10	—	1
2 : 9-Dimethyl-1 : 10-phenanthroline	200	—	—
1 : 10-Phenanthroline	200	—	—
Salicyaldehyde	300	—	10
Diphenylthiocarbazone	200	—	—
2 : 2'-Bipyridine	1000	—	—
Mercaptosuccinic acid	200	—	—

Succinate- and DPNH-cytochrome *c* reductase systems and the succinate dehydrogenase coenzyme Q system and the methods of measuring inhibition are given under Methods.

biologically-active inhibitors under consideration. Antimycin A inhibits 50 per cent at 5×10^{-8} M, *n*-heptyl-4-hydroxyquinoline-N-oxide at 5×10^{-7} M[3, 4] and the 2-hydroxy-3-alkyl naphthoquinones at 3×10^{-6} M.[6, 7] Greater inhibition by 2-thenoyl trifluoroacetone than by 2-furoyltrifluoroacetone, can be ascribed to stronger metal co-ordination by the thiophene sulfur than by the furan oxygen; 2-thenoyl trifluoroacetone forms tridentate chelates of great stability.[16, 17] The greater inhibition by the diphenyl phenanthrolines than by the other phenanthrolines appears to be related to their more lipophilic nature.

When the succinate reduction of the cytochromes *a*, *b* and *c*, in 2 mg of electron transport particle per ml, was measured spectrophotometrically in the presence of 1×10^{-4}M or 1×10^{-3} M 2-thenoyl trifluoroacetone or 5×10^{-4} M 4 : 7-diphenyl-1 : 10-phenanthroline, there was no evidence of specific inhibition between cytochromes *b* and c_1, as has been characteristically found for antimycin A, 2-heptyl-4-hydroxyquinoline N-oxide, and the alkylhydroxynaphthoquinones. Rather, there was a general inhibition of the reduction of all three cytochromes.

Some of the better inhibitors were tested for inhibition of the succinic dehydrogenase–coenzyme Q reaction and DPNH–cytochrome *c* reductase activity (Table 4). There was strongest inhibition of the succinic dehydrogenase–coenzyme Q reaction.

Reduction of internal coenzyme Q in mitochondria by succinate was inhibited by 5×10^{-4} M thenoyl trifluoroacetone. Thus, thenoyl trifluoroacetone is a good inhibitor of succinate-catalyzed reductions in the electron transport. Since its mechanism of inhibition must involve metal co-ordination, thenoyl trifluoroacetone should prove useful in future studies of the function of metals in electron transport. The major limitation is that the known co-ordination compounds studied here do not have the specificity of the inhibitors, antimycin A, 2-hydroxyl-3-alkylnaphthoquinones and 2-heptyl-4-hydroxyquinoline-N-oxide. The electron transport particle contains 32 moles of non-heme iron and 4 moles of copper per mole of flavin, and 35 per cent lipid,[11, 12] so there are many sites at which the lipophilic metal co-ordination compounds may inhibit. Their value will be greatest in detailed studies of purified preparations of electron transport chain components in which the number of possible inhibition sites is limited.

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PHARMACOLOGICAL PROPERTIES OF γ-AMINOBUTYRYLCHOLINE A SUPPOSED INHIBITORY NEUTROTRANSMITTER

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Abstract—The pharmacological properties of γ-aminobutyrylcholine (GABuCh) have been investigated on isolated organs and in the anaesthetized cat. This choline ester was found to be extremely inactive on traditional test organs as compared with acetylcholine. Given in moderate doses to the cat, GABuCh most consistently gave a fall in blood pressure and a neuromuscular block. The effect on heart rate was irregular. Only in extremely high doses was any effect observed on ganglionic transmission; this took the form of a slight ganglionic block. GABuCh in itself had negligible effects on the guinea pig ileum but sometimes exhibited slight antihistaminic and anticholinergic action on this test organ. No anti-5-hydroxytryptamine effect was demonstrable in the rat fundus preparation. GABuCh is hydrolysed at a very low rate by serum cholinesterase but not at all by human red cell cholinesterase. The results are discussed in connexion with other available data of GABuCh.

Ever since the first reports about a brain extract with inhibitory action upon the crayfish stretch receptor (Factor I)[1] there has been an intense search for the active component which could be assumed to be an inhibitory transmitter. The interest was first of all concentrated on γ-aminobutyric acid (GABA) which occurred in the inhibitory extract prepared by Bazemore *et al.*[2] and which since 1950[3] had been known to be a normal constituent of the mammalian brain. However, many but not all of the actions of Factor I could be reproduced by GABA. Further analysis of the inhibitory extract showed that other amino-acids like γ-guanidinobutyric acid might be responsible for some of the effects on the crayfish stretch receptor and other test organs. Another constituent of the active inhibitory extract might be γ-aminobutyrylcholine (GABuCh).[4] The presence of this ester in the brain was first pointed out by Kuriaki *et al.*[5] In an elaborate study, entirely unconcerned with Factor I, Kewitz has recently demonstrated beyond doubt the presence of GABuCh in considerable amounts in pig's brain.[6,7] Investigations of the effects of GABA and GABuCh on the cortical activity by Takahashi *et al.*[8] have shown that both substances are active in suppressing metrazol-induced spikes but that GABuCh is about 1000 times as active as GABA. The present study deals with the peripheral pharmacological effects of synthetic GABuCh.

EXPERIMENTAL

Compounds used

GABuCh as the chloride hydrochloride was obtained from L. Light & Co. Ltd., Colnbrook, England, and Riker Laboratories Inc., Northride, Calif. (the latter

kindly provided by Dr. Georg E. Cronheim). Also used were GABA (Light & Co.), acetylcholine iodide, butyrylcholine iodide, histamine dihydrochloride and 5-hydroxytryptamine creatinine sulphate (5-HT), (Hoffmann-La Roche & Co., Basel), suxamethonium iodide (Celocurin, Vitrum, Stockholm, Sweden) and hexamethonium bromide (Vegolysen, May & Baker Ltd., Dagenham, England). Choline chloride was kindly placed at our disposal by Dr. L. E. Tammelin, Research Institute of National Defence, Sundbyberg 4, Sweden. Nembutal, eserine salicylate and atropine sulphate were as described in the *Swedish Pharmacopoeia*, 11th Ed.

Recording of respiration, blood flow and pressure and muscular contractions

The effect of GABuCh was studied in more than twenty-five anaesthetized cats and a few unanaesthetized rabbits. Anaesthesia was induced with 5 per cent nembutal (0·8 ml/kg) intraperitoneally and maintained with further small doses of nembutal intravenously. Drugs were injected through a plastic cannula in the femoral vein of the cat and the marginal vein of the ear of the rabbit. Respiration, blood pressure and contractions of the gastrocnemius muscle and the nictitating membrane were recorded by passing the output of suitably arranged transducers into a Grass model 5 polygraph.

Respiration was recorded as pressure differences in the tracheal cannula by means of a pressure transducer (model PT 5, Grass Instrument Corporation, Quincy, Mass., U.S.A.).

Blood pressure was recorded with a Statham electromanometer from the left or right femoral artery.

The effect of drugs on neuromuscular transmission was determined by recording isometric contractions of the gastrocnemius muscle in response to supramaximal stimulation of the sciatic nerve. A force displacement transducer model FT 10, Grass Instrument Corporation, was used for recording the muscle contractions and the electrical stimuli were applied to the nerve distally to a crushed region by means of a Grass stimulator, model S 4, and shielded silver electrodes, with a duration of 5 msec and a frequency of 30/min.

The neuromuscular blocking effect of the drugs is expressed in terms of the PD_{50}, namely the dose required to produce 50 per cent blockade and the duration of such a block. To determine these quantities, the drugs were injected intra-arterially through a cannula in the femoral artery of the leg opposite to that being stimulated, the tip of the cannula lying proximally to the aortic bifurcation. The percentage diminution of twitch height and duration of block were recorded for at least three doses and the PD_{50} estimated by graphical interpolation.

Contractions of the nictitating membrane were recorded by means of a force displacement transducer FT 03. The cervical sympathetic trunk was dissected free from the vagus. The excitability of the membrane was tested by stimulating the preganglionic trunk for 5–10 sec every 2 min with rectangular pulses of the duration of 5 msec, frequency of from 5 to 10/sec before and after administration of drugs. An automatic timer was used for the stimulation.

Blood flow measurements. Blood flow was recorded by the method of Lindgren[9]. All injections were given in saline intra-arterially in a volume of 0·1 ml. Acetylcholine was used as a standard and saline as repeated controls. An especially designed ordinate writer (unpublished) allowed the recording of the blood flow directly on one of the channels of the Grass instrument.

Other pharmacological preparations

Frog rectus abdominis muscle preparations. The procedure was essentially that of Chang and Gaddum[10]. In this and the following preparation the activity of the esters was expressed as the relative molar potency as compared with acetylcholine.

Guinea-pig ileum preparations. A test bath of 4 ml volume was used. An automatic timer regulated the cycle of operations except for the addition of the compounds which was made manually in a small volume (0·1 ml) into the test bath by means of a microsyringe fitted with plastic tubing. The time of contraction was 30 sec and the different doses of GABA and GABuCh were added 50–60 sec before the stimulating drugs and in the resting intervals. The doses are given as micrograms added to the bottom of the test bath.

Rat fundus preparation. The influence of GABuCh on the 5-HT-induced contractions of the rat fundus was studied by the method described by Vane[11]. The volume of the test bath without fundus strip and oxygenation was 7 ml. The same automatic timer and means of addition as for the guinea pig ileum preparation were used. The cycle was that given by Vane. GABuCh in volumes 0·1—0·2 ml was added to the bottom of the test bath 20–60 sec before submaximal doses of 5-HT. Corresponding volumes of the incubation solution alone were added as controls before 5-HT.

Hydrolysis of esters by cholinesterases

The enzymic hydrolysis was measured electrometrically at 250 °C and pH 8 in barbitone buffer as described by Tammelin[12]. The following enzyme preparations were used: purified human plasma cholinesterase stated to correspond to Cohn's plasma fraction IV-6-3 (AB Kabi, Stockholm, Sweden), human erythrocyte cholinesterase prepared by haemolysing red cells with distilled water (final dilution 1:3) after repeated washing with saline solution, guinea pig brain homogenized in barbitone buffer and containing 0·23 g brain tissue/ml.

The hydrolysis was measured over a wide range of substrate concentrations using acetylcholine iodide, butyrylcholine iodide and GABuCh chloride hydrochloride. Enzyme concentrations were adjusted to give convenient rates of hydrolysis with the different substrates. Controls with inactivated enzymes were run simultaneously. Spontaneous hydrolysis never exceeded 1 per cent. The rate of hydrolysis is expressed as millimoles acid liberated per milligram enzyme preparation per minute. Hydrolysis of acetylcholine and butyrylcholine was calibrated by introducing known amounts of acetic acid into the reaction vessel instead of substrate by means of an Agla micrometer syringe (the pK's of acetic and butyric acid are sufficiently close for this calibration to be valid for both substrates). Calibration of the hydrolysis of GABuCh chloride hydrochloride was carried out by introducing known amounts of a mixture of GABA (0·2 M) and HCl (0·2 M) in equal amounts.

RESULTS

Unanaesthetized rabbits

At repeated doses of 0·5–1·0 mg GABuCh/kg there were no objective symptoms in the rabbit. At 2 mg there was a distinct decrease in the frequency and amplitude of respiration and the animal appeared to feel ill. All symptoms occurred within 30 sec

after the injection. At 4 mg per kg a further decrease in the rate of respiration occurred which gave a distinct peripheral cyanosis. Some salivation was observed. There was a successive paralysis of the muscles with a head drop within 3 min. Even after 10 min the rabbit was incapable of standing on its feet. At 15 min the respiration was normal again and at 20 min the animal was able to walk around slowly on the floor. On the following days the animals did not show any symptoms of illness. Thus, in the unanaesthetized rabbit at least two objective symptoms are clearly visible, namely depression of respiration and paralysis of the skeletal muscles.

Anaesthetized cats

Effects on respiration. As seen in Figs. 1 and 2, after small doses which did not markedly affect the neuromuscular transmission, there were sometimes single gasps immediately after the injection. Equimolar doses of GABA always stimulated the respiration. At higher doses (2 mg or more) there was a distinct decrease in the amplitude and frequency of respiration. The dose which gave apnoea varied between 3 and 10 mg/kg with big individual fluctuations. The cats always survived after a short period of artificial respiration.

Effects on blood-pressure and heart rate. GABuCh produces a fall in the arterial blood pressure (Figs. 1 and 2) when given intravenously to cats. The depressor effect is first evident at doses of 0·2 mg/kg. At 1 mg/kg the average fall in blood-pressure is about 20 per cent. There could sometimes be observed two phases in the blood-pressure fall, one immediate and short-lasting phase and another longer-lasting phase with a very slight decrease. Atropine in different doses (0·5–4 mg/kg) did not abolish or reverse the depressive effect of GABuCh on the blood pressure. However, before atropine the fall was more pronounced. GABA in equimolar doses sometimes caused a moderate decrease, sometimes a slight increase of the blood pressure.

The effect of GABuCh on the heart rate was more variable. In general there was an immediate decrease in the heart rate after the injection. However, in a few cats an acceleration of the pulse rate was observed and in other cats the effects were negligible. Bradycardia occurring could be inhibited by atropine (Fig. 2).

Effect on nictitating membrane. The effect of GABuCh on ganglionic transmission in the superior cervical ganglion as recorded by the contractions of the nictitating membrane after preganglionic sympathetic stimulation is almost negligible. Systemic intravenous doses from 1 to 10 mg do not produce any demonstrable ganglionic block (Fig. 1). From the figure it can be seen that there is a slight elevation of the base line of the nictitating membrane at the injection of GABuCh. However, this effect persists after hexamethonium and extirpation of the ganglion and gives no simultaneous potentiation of the contractions. This effect is possibly due to the influence of the drug on the external muscles of the eye. At extremely high doses there are effects on the ganglionic transmission. At 1 mg given directly into the artery supplying the ganglion there is a rapidly disappearing ganglionic block (Fig. 3).

Effect on neuromuscular transmission. GABuCh has neuromuscular blocking effects (Fig. 1) in doses above 0·5 mg/kg intravenously. The block is intensified by eserine and preceded by fasciculations, suggesting a depolarizing type of block. The duration of the block at doses of 1 to 2 mg was about 10 to 15 min. The PD_{50} was estimated to 65 μg/kg as compared with 3 μg/kg for suxamethonium (Table 1). The duration of the GABuCh block was a little longer.

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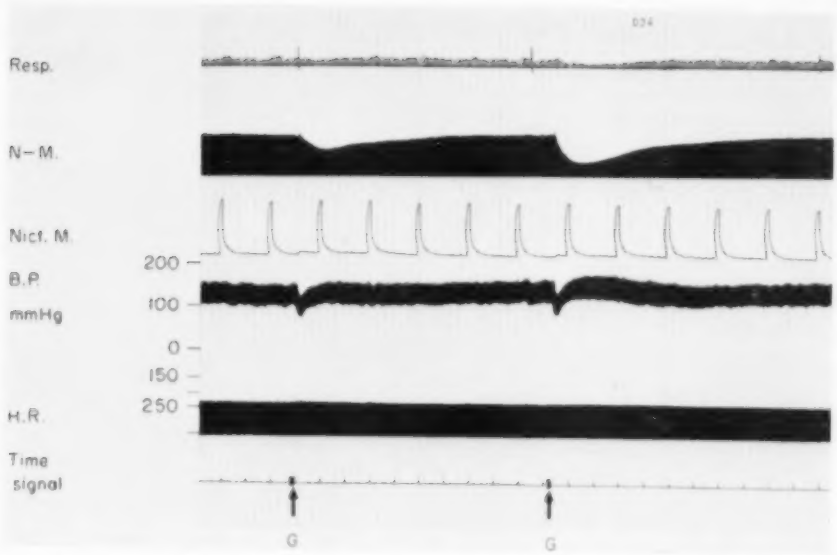

FIG. 1. Effect of GABuCh (G) on respiration (RESP.), neuromuscular transmission (N–M.), nictitating membrane, (NICT.M.), blood pressure (B.P.) and heart rate (H.R.). Abscissa, time in min. Cat 1·7 kg. Anaesthetic, nembutal. Heart rate in beats/min. (scale inverted). First injection, 1·5 mg/kg., second injection 2·0 mg/kg G.

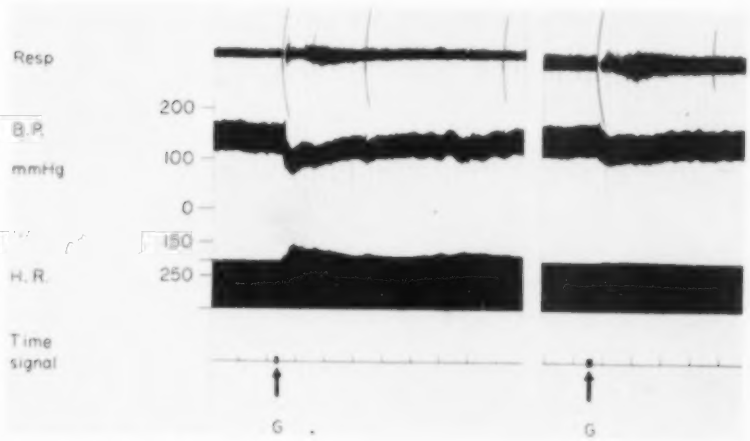

FIG. 2. Effect of GABuCh on respiration, blood pressure and heart rate. Abbreviations, units and anaesthetic as in Fig. 1. Cat 2·1 kg. Dose, 1 mg/kg. First injection before and second injection after atropine (2 doses of 2 mg/kg).

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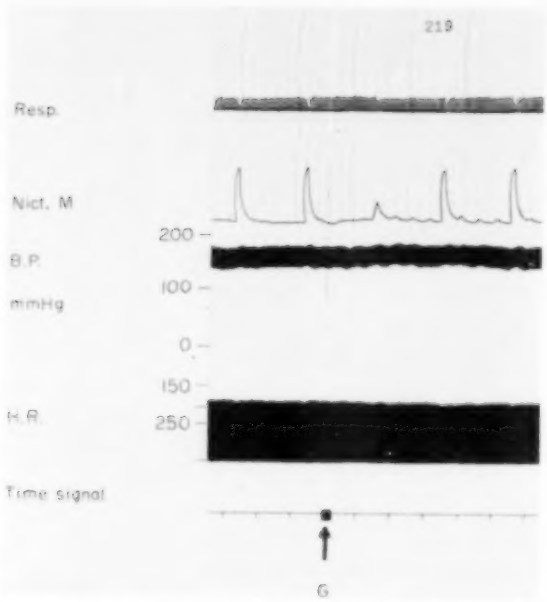

FIG. 3. The influence of GABuCh on the ganglionic transmission. Abbreviations, units and anaesthetic as in Fig. 1. Cat 2·2 kg. Dose, 1·0 mg injected into the external carotid artery, the branches of which except those to the superior cervical ganglion had been ligated. Stimulation of the isolated central stump of the sympathetic trunk for 10 sec every 2 min. with rectangular pulses of 5 msec. duration, 3 V amplitude and at a frequency of 5/sec.

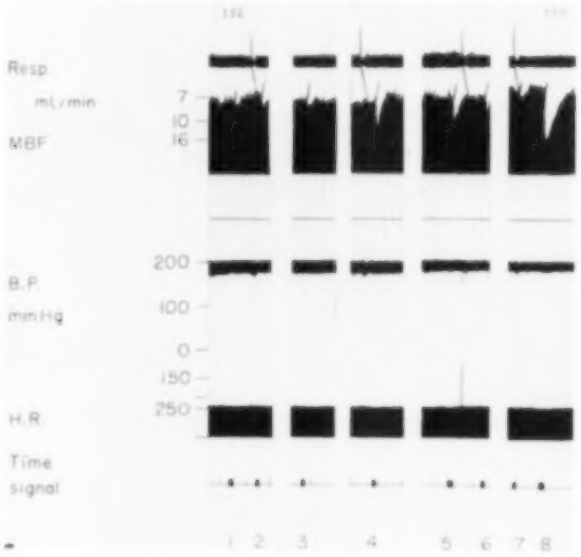

FIG. 4. Effect of GABuCh compared with that of acetylcholine on the muscular blood flow (MBF) in the left hind limb recorded in the femoral artery. Other abbreviations, units and anaesthetic as in Fig.1. Cat 3·3 kg. The following injections were made intraarterially in 0·1 ml saline: (1) saline alone; (2) to (4) GABuCh, 1, 100 and 1000 μg; (5) saline immediately after (4); (6, 7) saline; (8), acetylcholine 0·01 μg.

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TABLE 1. ACTIVITY OF GABuCh ON FROG RECTUS ABDOMINIS MUSCLE, GUINEA-PIG ILEUM AND CAT SCIATIC NERVE-GASTROCNEMIUS MUSCLE PREPARATION

Ester	Relative molar potency		
	Frog rectus	Guinea-pig ileum	PD_{50} Cat sciatic-gastrocnemius preparation
Acetylcholine	100	100	—
GABuCh	0·1	0·05	2·7
Succinylcholine	—	—	100

Effects on peripheral blood flow. When compared with acetylcholine, which has a well-known peripheral vasodilator effect, GABuCh shows an extremely weak and nonspecific one (Fig. 4). At doses of 1 mg intra-arterially there is a demonstrable increase in the muscular blood flow of the leg. Even this high dose had no effect whatsoever on the blood flow in the superior mesenteric artery.

Activity on frog rectus and guinea pig ileum

The nicotinic action was stronger than the muscarinic action. However, both were extremely low (Table 1). A typical response curve of the frog rectus is seen in Fig. 5.

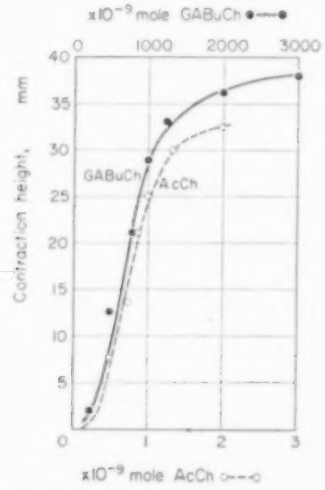

FIG. 5. The effect of GABuCh on the frog rectus preparation compared with acetylcholine. Abscissa doses in mμ moles.

Investigations concerning possible inhibitory effects on the action of smooth muscle stimulating drugs

The possible inhibitory effect of GABuCh was studied on the guinea pig ileum (histamine and acetylcholine) and on the rat fundus strip (5-HT). In highly-sensitive intestines (reacting to from 0·001 to 0·01 μg histamine base and 0·2 μg acetylcholine) the addition of from 0·1 to 10 μg GABuCh *sometimes* achieved an inhibitory effect

amounting to about 50 per cent of the contraction height. Generally this effect could not be repeated on the same intestine. Doses above 10 μg of either had no effect at all or gave a slight potentiation of the contraction.

The 5-HT contraction of the fundus strip was not significantly modified by doses of GABuCh from 1 to 100 μg. In higher doses the 5-HT contraction was slightly potentiated (Figs. 6–7), however,not more than by equimolar doses of choline. Nor were the contractions of GABuCh alone greater than those of choline. GABA itself had no inhibiting effect on this test preparation.

Enzymic hydrolysis

The results of the studies of enzymic hydrolysis are evident from Fig. 8. It will be seen, that a highly-purified preparation of butyro-cholinesterase hydrolyses GABuCh very slowly compared with butyrylcholine and acetylcholine. Erythrocytes did not hydrolyse GABuCh at all. Neither did the fairly-concentrated homogenate from rabbit brain produce any significant degree of hydrolysis of the GABuCh, the rate relative to acetylcholine being about 1 per cent.

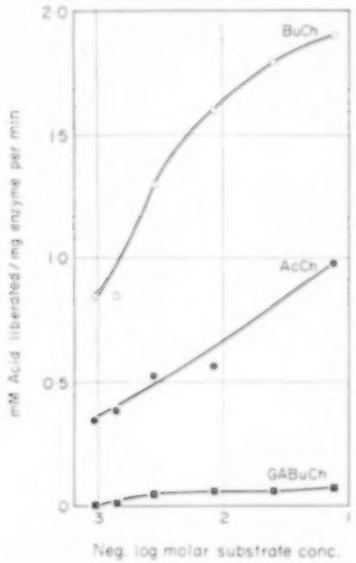

Fig. 8. Hydrolysis of butyrylcholine (BuCh), acetylcholine, (AcCh) and GABuCh by a purified preparation of butyrylcholinesterase.

DISCUSSION

As mentioned in the introduction, GABA has by some investigators been proposed as an inhibitory transmitter. Others do not agree with this (Curtis *et al.*[13]). From investigations made on the crayfish stretch receptor and brain of mammals are to be distinguished experiments on peripheral organs for which no inhibitory nerve supply is known (Hobbiger[14,15]).

From some recently-published investigations it would seem as though the choline ester GABuCh is much more active as an inhibiting compound than GABA[4,8] GABA is known to occur in large amounts in the central nervous system. The

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FIG. 6. The effect of GABA and GABuCh on 5-HT contractions of the rat fundus preparation.
(S) 2 mμg 5-HT (mμg = 10^{-9} g).
(1, 2, 3) 10,100 and 1000 μg GABA followed by 2 mμg 5-HT.
(A, B, C) 10,100 and 1000 μg GABuCh followed by 2 mμg 5-HT.
(G) 1000 μg GABuCh alone.
(O)blank = background contraction of Vane preparation with no drug added.

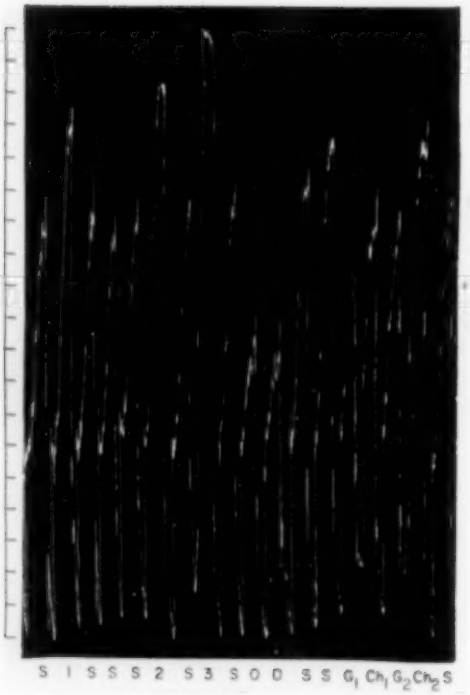

FIG. 7. The effect of GABuCh and choline in equimolar concentrations on 5-HT contractions of the rat fundus preparation.

(S): 0·5 mμg 5-HT.

(1): 0·2 ml 10^{-2}M choline. (2) 0·1 ml 10^{-2}M GABuCh. (3) 0·1 ml 10^{-2}M choline, all followed by 0·5 mμg 5-HT.

(O): blank.

(G_1 and Ch_1): 0·05 ml 10^{-2}M GABuCh and choline, respectively.

(G_2 and Ch_2): 0·1 ml 10^{-2}M GABuCh and choline, respectively.

identification of GABuCh in comparatively large amounts in brain arises many questions. It is the third choline ester positively to have been identified in mammalian tissue and the second one to have been found in brain.[16] It might constitute the naturally-occurring substrate for butyro-cholinesterase, the role of which is still unknown;[17] however, the slow rate of hydrolysis of GABuCh in comparison with butyryl- and acetylcholine by both purified butyro-cholinesterase and brain tissue presented in this paper, makes this hypothesis less likely.

Previous publications on the action of GABuCh on pharmacological test organs are few. Our own results disclose surprisingly few peripheral effects of this choline ester when given in reasonable doses. In summary, the cardiovascular effects consist of a blood pressure fall and a slight bradycardia. The fall in blood pressure cannot be explained by peripheral vasodilatation since the doses used did not effect the peripheral blood flow of the leg muscles (Fig. 4) or intestinal canal; furthermore, it cannot be explained by a ganglion-blocking action of the ester, since this was seen only at very high doses (Fig. 3) and the fall in blood pressure also occurs after ganglion-blocking doses of hexamethonium.

Compared to GABA[18] the cardiovascular effects of its choline ester are more pronounced and more consistent. GABA produced either a slight rise or a fall in blood pressure, whereas GABuCh invariably gave a fall in blood pressure, resistant to atropinization. Bradycardia accompanying this fall was, however, susceptible to atropine. That the cardiovascular effects produced were due to contaminants of the substances used is unlikely because compounds from two different sources produced the same effects. Besides having cardiovascular effects, GABuCh is a neuromuscular blocking agent with a potency of about 1/50 of that of suxamethonium. Kuriaki *et al.*[5] have reported a complete block by GABuCh of the histamine-induced contraction of the guinea pig ileum. The antihistaminic effect was evident only over a very narrow dose range. The present writers, however, could never find such a strong antihistaminic potency of the choline ester. Even in intestines reacting to doses as small as 0·001 μg histamine never more than a 50 per cent inhibition was demonstrated and even this effect was very irregular. The weak antihistaminic effect often appeared and disappeared successively.

GABA has been reported to have antihistaminic, antiacetylcholine and anti-5-HT effects on the guinea pig intestine[14,15] in doses from 10 to 100 μg/ml. At submaximal doses of histamine and acetylcholine the inhibiting effect of GABA amounted to 35–75 per cent. The anti-5-HT effect, however, was more pronounced (75–95 per cent.). On other test organs, such as the rat duodenum, Hobbiger could not find as convincing results as with the guinea pig ileum. This species variation is of interest when compared with our completely negative results of both GABA and GABuCh on the rat fundus preparation, an extremely sensitive test organ for 5-HT.

In the guinea pig ileum Whittaker[19] has observed an anti-5-HT effect of GABuCh similar to the antihistamine and antiacetylcholine effects reported here. These irregular and evanescent inhibitions remain to be further investigated; however, it is already clear that they are unlikely to provide a basis for the pharmacological assay of GABuCh.

With reference to the antagonism between GABuCh and histamine it can be noted that Dale[20] demonstrated an inhibitory effect of acetylcholine on the response of the guinea pig ileum to histamine when the two drugs were repeatedly added to the test bath. When the response to small doses of histamine had disappeared the contraction

could be elicited by larger doses. This is similar to our findings and to the investigations of Kuriaki *et al.*[5].

The relative inactivity of GABuCh on all the peripheral test organs studied is somewhat distressing. Two things, however, must be born in mind, firstly, that the possible action in the central nervous system is not imitated by these experiments and, secondly, that peripheral inhibitory nervous pathways up till now have not been found in mammals.

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EFFECTS OF HYPOGLYCIN ON CERTAIN ASPECTS OF GLUCOSE AND FATTY ACID METABOLISM IN THE RAT

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Abstract—Hypoglycin (2-amino-methylene*cyclo*propanepropionic acid), isolated from the seeds of *Blighia sapida*, induced a marked hypoglycemia when administered to rats. Little direct effect of this compound was observed on glucose metabolism, either on glucose oxidation to respiratory CO_2 or on conversion of glucose to liver lipid or muscle glycogen. Synthesis of glycogen from glucose in diaphragm muscle was inhibited by hypoglycin without altering glucose uptake.

The major effects of hypoglycin were those concerned with fatty acid metabolism. The administration of hypoglycin to rats caused an increase in the level of non-esterified fatty acids in the serum, an increase in the total lipids of the liver and a significant inhibition in the conversion of butyrate or stearate to respiratory CO_2. Liver mitochondria isolated from hypoglycin-treated rats showed an impaired ability to form high energy phosphate bonds associated with the oxidation of pyruvate and malate. Hypoglycin had no effect on adrenal cortical output.

Since hypoglycin lowered blood glucose level only after 3 or 4 hr, the active compound may be a metabolite or the effects on blood sugar may be secondary to some primary metabolic block. Methylene*cyclo*propanepyruvic acid and methylene*cyclo*propaneacetic acid, both likely metabolites, do produce hypoglycemia. The possibility that such intermediates interfere with the oxidation of fatty acid substrates is briefly discussed.

INTRODUCTION

Hypoglycin, a compound isolated from the seeds of *Blighia sapida*,[1,2] is capable of inducing hypoglycemia in several animal species. This compound has aroused considerable interest recently as a result of the elucidation of its structure as α-amino-methylene*cyclo*propanepropionic acid.[3–6] Although studies have been reported on certain metabolic effects of hypoglycin, its mode of action in inducing hypoglycemia remains obscure.

A primary metabolic effect of hypoglycin has been postulated to be an inhibition of liver glycogen formation.[1,7] On the other hand, recent experiments in our laboratory demonstrated the ability of hypoglycin to induce lipid infiltration in the liver and to produce a several-fold increase in the serum non-esterified fatty acid (NEFA) level in intact rats.[8] Also, on the basis of histological evidence, Chen *et al.*[9] reported fat infiltration in the livers of hypoglycin-treated rats. Further investigation into the effect of hypoglycin on fatty acid metabolism therefore appeared to be of interest.

This paper describes the effects of this interesting hypoglycemic agent on certain aspects of glucose and fatty acid metabolism. A comparison of some of these effects with those produced by insulin or tolbutamide (N-*p*-tosyl-N′-*n*-butyl urea) is also presented.

EXPERIMENTAL

Materials

Crystalline zinc-insulin was provided by Dr. O. K. Behrens of Eli Lilly and Company. Tolbutamide was purchased from the Upjohn Company. Hypoglycin was isolated from the seed of *Blighia sapida* by Dr. R. S. de Ropp and shown to be analytically pure. Mr. J. C. Van Meter prepared the methylene*cyclo*propanepyruvic acid by enzymatic deamination of hypoglycin and methylene*cyclo*propaneacetic by decarboxylation of this pyruvic acid derivative. Dr. S. Safir provided the 4-pentenoic acid. Radioactive glucose and fatty acids were purchased from the New England Nuclear Corporation.

Assays

Blood glucose,[10] liver and muscle glycogen[11] and liver total lipid[12] were determined by published procedures. Glucose uptake and glycogen synthesis were determined in rat diaphragm as described previously.[13] Adrenal cortical secretion was measured by the method of McKerns and Nordstrand.[14,15] R.Q. was determined on paired liver slices by the direct method of Dixon[16] using the Krebs–Ringer phosphate medium described by Umbreit *et al.*[17] Oxidative phosphorylation was studied in liver mitochondria using pyruvate and malate as substrates by methods similar to those described in Brody and Bain.[18]

Metabolism experiments with ^{14}C-labeled glucose or fatty acids

After the intramuscular administration of the hypoglycemic agent under study, 3–4 μc of glucose-U-^{14}C was injected intramuscularly, along with unlabeled glucose (1 g/kg body weight), either to fed or 20-hr fasted albino rats weighing about 100–150 g. Immediately thereafter, the rats were placed in a respiratory apparatus and the expired CO_2 was swept by filtered CO_2-free air into carbonate-free 20% sodium hydroxide. The sodium hydroxide was changed at $\frac{1}{2}$ hr intervals during a 2- or 4-hr experimental period and the CO_2 collected as $BaCO_3$. The radioactivity of the $BaCO_3$ precipitates was determined and the $BaCO_3$ weighed to determine total CO_2 excretion. At the end of the experimental period the rats were decapitated and selected tissues removed, weighed and subjected to the desired analysis.

The effect of hypoglycin on the metabolism of 1-^{14}C labeled fatty acids in the intact rat was studied in the following manner: 1-^{14}C-labeled acetate, butyrate or stearate was administered at a desired time after the intramuscular injection of hypoglycin. The fatty acids were injected either as saline, rat plasma or albumin suspensions. The route of administration was varied. In certain experiments non-labeled glucose was also administered intramuscularly along with the fatty acids. Respiratory CO_2 was collected and tissue analyses were performed as described below.

The metabolsim of radioactive fatty acids or glucose in liver slices was studied using the K-110 medium of Ashmore *et al.*[19] One hundred mg of tissue were incubated in 3 ml of medium for 1 hr in a modified Warburg flask containing a side arm closed with a rubber policeman; 0·4ml of 30% NaOH was then injected into the center well, followed by injection of 0·2 ml of 8 N H_2SO_4 into the main compartment. After allowing 2 hr for absorption of CO_2, the NaOH was removed and the center well washed out with 5 ml of CO_2-free water. The CO_2 was recovered as $BaCO_3$ by the addition of 0·5 ml of a solution of $BaCl_2$ (2 N) and NH_4Cl (1·6 N). The $BaCO_3$ was washed with CO_2-free water and finally with ethanol–ether (3:1).

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Radioactivity measurements

All radioactivity measurements were made on samples sufficiently thin that no corrections for self-absorption had to be made. A gas-flow counter with automatic sample changer was used. Samples were counted sufficiently long that a counting error of not more than 5 per cent was ensured.

RESULTS

Effect of hypoglycin, tolbutamide or insulin on blood glucose and liver glycogen levels in rats

Fig. 1 shows typical changes in the level of blood glucose after the administration of hypoglycemic agents to fasted rats. All rats received an intramuscular dose of 1 g

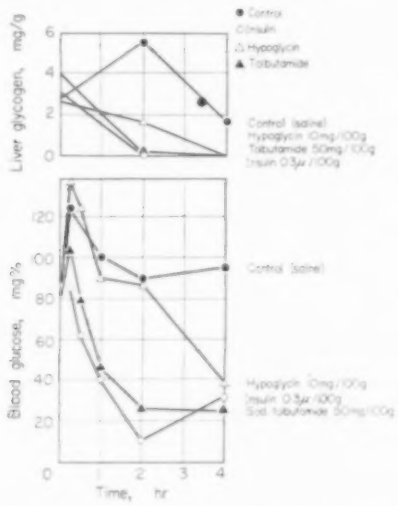

FIG. 1. Effect of hypoglycemic agents on the blood glucose and liver glycogen levels in the rat. Fasted rats were given the drug intramuscularly, followed by glucose (100 mg/100 g).

of glucose per kg of body weight along with the hypoglycemic agent. Both insulin and tolbutamide caused a rapid lowering in the level of blood glucose. The levels of blood glucose after hypoglycin-treatment followed the same pattern as the control for the first 2 hr, but dropped to low levels during the next 2 hr. Similar changes in the blood glucose levels were seen in other experiments after the intraperitoneal injection of hypoglycin. The associated changes in the levels of liver glycogen are also shown in Fig. 1. The liver glycogen level rose only in the case of the controls. The maximum rise was observed within 2 hr.

Effect of in vivo *administration of hypoglycin, tolbutamide or insulin on oxidative phosphorylation in liver mitochondria*

Since hypoglycin treatment caused a rapid infiltration of lipid into the liver,[8] it appeared of interest to determine oxidative phosphorylation capacity of liver mitochondria isolated from hypoglycin-treated rats. In these experiments the hypoglycemic agents were administered to starved rats which were killed by decapitation 4 hr later. The livers were excised and mitochondria isolated. Malate and pyruvate were used as

substrates. The results are shown in Table 1. A significant decrease in P:O ratio was observed only in the case of hypoglycin-treatment. When added *in vitro* to mitochondria prepared from untreated rats, hypoglycin had no effect (data not shown).

TABLE 1. OXIDATIVE PHOSPHORYLATION* IN LIVER MITOCHONDRIA PREPARED FROM FASTED RATS FOUR HOURS AFTER TREATMENT WITH HYPOGLYCIN (15 mg/100 g), TOLBUTAMINE (100 mg/100 g), OR INSULIN (0·2 U/100 g)

Expt. no.	Mitochondria from	N per flask (mg)	Pi† uptake (μ moles)	O_2 uptake (μ atoms)	P : O‡ control	P : O‡ tested
			Mean of 3 Flasks			
1	Control	4·4	19·7	7·6	2·61	2·04
	Hypoglycin	4·3	12·2	7·2		
2	Control	4·4	15·7	7·8	$t = 3{\cdot}62$	
	Hypoglycin	4·4	12·4	7·7		
3	Control	3·8	33·2	10·5	$P < 0{\cdot}02$	
	Hypoglycin	3·7	26·5	9·6		
4	Control	4·6	16·2	8·5	2·20	2·27
	Tolbutamide	4·6	14·3	7·4		
5	Control	4·2	21·9	8·6		
	Tolbutamide	3·8	23·1	9·0		
6	Control	4·8	25·9	12·0	$t =$ NS	
	Tolbutamide	4·5	29·6	13·2		
7	Control	5·4	22·5	10·2	2·23	2·40
	Insulin	4·1	21·8	9·0		
8	Control	4·0	13·0	6·1	$t =$ NS	
	Insulin	4·0	10·1	6·4		
9	Control	4·0	19·3	8·3		
	Insulin	3·8	18·6	7·8		

* Determined as described by Brody and Bain.[18] Substrate concentrations: sodium pyruvate, $1{\cdot}5 \times 10^{-2}$M; sodium malate, 2×10^{-3}M.
† Pi = Inorganic phosphate.
‡ Calculated from the average of the three experiments shown.

Effect of hypoglycin on the conversion of ^{14}C-labeled glucose or fatty acids to CO_2 in intact rats

The effect of hypoglycin on the conversion of glucose-U-^{14}C and acetate-1-^{14}C to CO_2 was determined at $\frac{1}{2}$ hr intervals following the administration of the ^{14}C-labeled compound. As shown in Table 2, no significant differences in $^{14}CO_2$-output from glucose-U-^{14}C were observed between treated and normal animals.

The conversion of acetate-1-^{14}C to respiratory CO_2 in starved rats was slightly lower after hypoglycin-treatment. The simultaneous administration of glucose reduced this slight difference. Acetate conversion to CO_2 was not affected in fed rats (data not shown). The slight effect on acetate metabolism in fasted rats was probably a reflection of the very low carbohydrate reserves. The total respiratory CO was reduced in all

cases. The total $^{14}CO_2$ (expressed in counts/min per 100 g of body weight) and total respiratory CO_2 at the end of the experiments are shown in Table 2.

In contrast to the results with acetate, the conversion of butyrate-1-^{14}C to $^{14}CO_2$ in

TABLE 2. THE EFFECT OF HYPOGLYCIN ON THE CONVERSION OF ^{14}C-LABELED GLUCOSE OR ACETATE TO CO_2 IN INTACT RATS

Exp. no.	Glucose-U-^{14}C				Acetate-1-^{14}C			
	1		2		3		4	
	Control	Hypoglycin (10 mg/100 g)	Control	Hypoglycin (15 mg/100 g)	Control	Hypoglycin (15 mg/100 g)	Control	Hypoglycin (15 mg/100 g)
Counts/min per 100 g	328,850	345,968	364,947	419,160	1,248,418	1,072,849	2,213,957	2,095,026
ml CO_2 per 100 g body weight	1021	636	358	268	333	195	386	264

Glucose-U-^{14}C, 4 μc/100 g, was administered intramuscularly with non-labeled glucose (100 mg/100 g) to fasted rats in exp. 1; 3 μc to fed rats in exp. 2.

Acetate-1-^{14}C, 7 μc/100 g, was administered intramuscularly to fasted rats in exp. 3; acetate-1-^{14}C (7 μc) with non-labeled glucose (100 mg/100 g) to fasted rats in exp. 4.

The radioactive compounds were given 4 hr after hypoglycin in exp. 1 and 5 min after hypoglycin in exp. 2, 3 and 4. CO_2 was collected for 4 hr in exp. 1 and for 2 hr in exp. 2, 3 and 4. Values shown are total accumulative ^{14}C-output at these times.

Data shown are for individual rats and are representative of at least two rats on each treatment.

fed rats was found to be greatly reduced when it was administered intravenously 4 hr after the administration of hypoglycin. In these experiments, butyrate-1-^{14}C was administered as a neutralized suspension with bovine serum albumin. The results of two separate experiments are given in Fig. 2. The most striking difference between the control and the hypoglycin treatment was seen in the first $\frac{1}{2}$ hr after the administration of the butyrate.

Similarly the conversion of stearate-1-^{14}C to $^{14}CO_2$ in fed animals which received hypoglycin was also reduced. Stearate was administered intramuscularly as a rat plasma suspension. Results are shown in Fig. 3.

Effect of hypoglycin on incorporation of ^{14}C-labeled fatty acids into liver lipid in vivo

Previous experiments demonstrated a marked rise in serum NEFA level and moderate increase in liver total lipid following hypoglycin-treatment.[8] It was of further interest to study the effect of hypoglycin on incorporation of ^{14}C labeled substrates into liver lipids *in vivo*. These data are shown in Table 3 along with data on the conversion of ^{14}C labeled glucose into liver lipid. They show that hypoglycin treatment had no effect on the conversion of these substrates to liver total lipid. However, specific activities were somewhat lower since the total lipids were higher. This suggests a mobilization of lipid from body stores.

Effect of hypoglycin and related compounds on conversion of glucose U ^{14}C *or acetate-1-^{14}C to CO_2 by liver slices*

In these experiments labelled glucose or acetate was incubated alone or with the

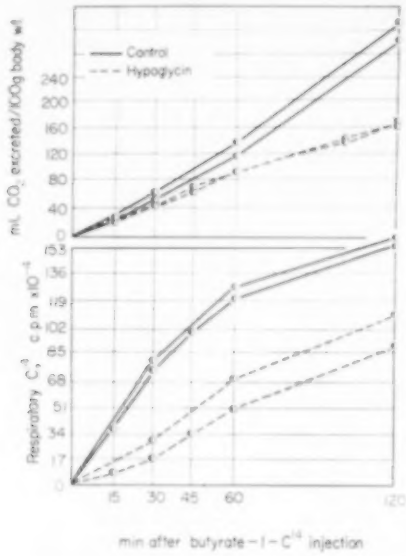

FIG. 2. Effect of the administration of hypoglycin on the metabolism of butyrate in the intact rat. Butyrate-1-^{14}C was administered intramuscularly to fed rats 4 hr after the intramuscular injection of hypoglycin. Each value is from a single rat.

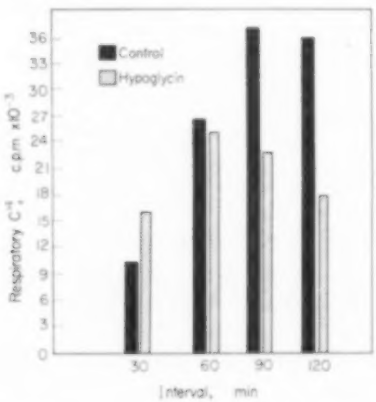

FIG. 3. Effect of hypoglycin on the excretion of $^{14}CO_2$ derived from stearate-1-^{14}C. The fatty acid was injected intramuscularly as a plasma emulsion 5 min after the intramuscular administration of hypoglycin (15 mg/100 g). Each value is from a single rat.

compound under study with liver slices obtained from fasted rats. Incorporation of ^{14}C into CO_2 was measured. The results are shown in Table 4. As expected, non-labeled acetate suppressed the conversion of acetate-1-^{14}C to CO_2. At the concentration employed, no marked effect on the conversion of either glucose or acetate to CO_2 was observed with hypoglycin, methylene*cyclo*propanepyruvic acid or methylene-*cyclo*propaneacetic acid. A slight stimulation of glucose conversion to $^{14}CO_2$ was observed in the presence of the latter compound, but further experiments must be done

TABLE 3. THE EFFECT OF THE ADMINISTRATION OF HYPOGLYCIN (15 mg/100 g) FOLLOWED BY ^{14}C-LABELED SUBSTRATES ON THE INCORPORATION OF ^{14}C INTO THE LIVER LIPIDS OF THE RAT

Exp. no.	Total counts/ min per liver	mg of lipid/ g liver	Liver wt. (g)	Total counts/ min per liver	mg of lipid/ g liver	Liver wt. (g)
Glucose-U-^{14}C						
1	9831	62·7	3·61	10,672	70·0	3·84
2	8740	56·5	5·27	7561	63·5	3·71
3	5865	59·9	2·04	6171	50·5	2·05
4	13,966	56·6	4·70	15,727	64·9	5·06
	9406	54·6	3·87	10,559	62·4	4·8
5	2460	56·3	2·79	3153	73·7	2·24
	7765	61·0	2·11	3316	78·7	2·03
Acetate-1-^{14}C						
6	4936	54·4	3·50	5512	65·2	3·95
7	5772	53·7	4·01	5610	72·2	4·17
Butyrate-1-^{14}C						
8	6176	59·3		7935	69·3	4·67
9	7867	55·1		7866	68·0	4·84

Acetate-1-^{14}C or Glucose-U-^{14}C (4 μc/100 g) administered intramuscularly with non-labelled glucose (50 and 100 mg/100 g, respectively) to fasted rats.

Butyrate-1-^{14}C (4 μc/100 g) bound to albumin administered intravenously to fed rats.

The radioactive compounds were administered 2 hr (exp. 1–4) or 4 hr (exp. 6–9) after hypoglycin. Liver lipids were measured 2 hr after the administration of ^{14}C-labeled compound.

In expt. 5 the glucose-U-^{14}C was administered 5 min after hypoglycin and the liver lipids were measured 4 hr later.

TABLE 4. THE EFFECT OF HYPOGLYCIN AND ANALOGS ON THE CO_2-PRODUCTION OF LIVER SLICES FROM GLUCOSE-U-^{14}C OR ACETATE-1-^{14}C *in vitro*

Substrate	Control	Hypo-glycin	2-Methylene *cyclo*propane pyruvic acid $CH_2 = \Delta\ CH_2COCOOH$	2-Methylene *cyclo*propane acetic acid $CH_2 = \Delta\ CH_2COOH$	Acetic acid CH_3COOH	4-Pentenoic acid $CH_2 = CHCH_2CH_2COOH$
Glucose-U-^{14}C 0·1 μc/ml	355 ±56	263 ±15	377 ±61	539		
Acetate-1-^{14}C 0·1 μc/ml	3299 ±19	2866 ±21	3470 ±31	3040 ±23		
	8994 ±18				5253 ±36	3174 ±48

Incubations were for 1 hr in K-110 buffer[19] containing 0·2% glucose, the substrate shown and 10^{-3} M of hypoglycin or analog. Values are counts/min per 100 mg tissue (±5·0) and the mean of three determinations. They represent metabolic CO_2 derived from the substrate.

in order to determine the significance of this finding. 4-Pentenoic acid significantly inhibited acetate-1-^{14}C conversion to CO_2. This compound has been reported to cause hypoglycemia in rats.[20]

Effect of hypoglycin on glucose uptake and glycogen deposition in rat diaphragm

Diaphragm from untreated or hypoglycin-treated rats was compared in these experiments. Hypoglycin was also added *in vitro* to diaphragm removed from untreated

rats. Results are shown in Table 5. No significant effect on glucose uptake was observed either when hypoglycin was added *in vitro* or when administered *in vivo*. It is interesting, however, to note the significant inhibition of net glycogen formation induced by hypoglycin both *in vivo* and *in vitro*.

TABLE 5. EFFECT OF *in vivo* PRETREATMENT WITH HYPOGLYCIN ON THE SUBSEQUENT GLUCOSE UPTAKE AND GLYCOGEN FORMATION OF RAT HEMIDIAPHRAGM

(Starved rats were decapitated 4 hours after injection. Insulin at 1 milliunit/ml. Results expressed as mg/g of diaphragm).

	Control		Hypoglycin	
	U	S	U	S
Glucose uptake	3·14	5·00	3·43	4·95
		s.e. m. = ±0·29		
Glycogen formation	2·56	3·50	1·91	2·52
		s.e. m. = ±0·17		

	Glucose uptake	Glycogen formation
t between U and S of control =	4·54†	3·92†
t between U and S of hypoglycin =	3·71*	2·54*
t between U and U =	NS	2·71*
t between S and S =	NS	4·08*

Each value is the mean of four determinations.
U = unstimulated.
S = stimulated by insulin.
* = $P < 0.01$.
† = $P < 0.001$.
NS = not significant.

Effect of hypoglycin on R.Q. of rat liver slices

The effect of hypoglycin on R.Q. of liver slices prepared from hypoglycin-treated rats is shown in Table 6. It can be seen that the R.Q. was slightly lowered due to a higher QO_2 when hypoglycin was administered 4 hr prior to the removal of the liver.

TABLE 6. THE EFFECT OF HYPOGLYCIN (10 mg INTRAMUSCULARLY/100 g) ON THE SUBSEQUENT R.Q. OF RAT LIVER SLICES *in vitro*

	Control			Hypoglycin		
	QCO_2	QO_2	R.Q.	QCO_2	QO_2	R.Q.
Exp. A (2 hr) Means (12)	2·6	4·3	0·61	2·8	4·4	0·64
			No significant differences			
Exp. B (4 hr) Means (12)	3·1	4·8	0·65	3·4	5·6	0·60

t between control and hypoglycin for QCO_2 = NS
t between control and hypoglycin for QO_2 = 3·7*
t between control and hypoglycin for R.Q. = 2·32*
* $P < 0.05$

Slices were incubated in Krebs–Ringer phosphate medium. In expt. A the livers were removed 2 hr after hypoglycin-treatment. In expt. B the livers were removed 4 hr after hypoglycin-treatment.

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No significant effect was apparent 2 hr after administration of hypoglycin (experiment A).

Effect of hypoglycin on adrenal corticoid secretion

It was conceivable that the pituitary–adrenal axis was involved in some of the metabolic changes brought about by hypoglycin. In order to investigate this possibility, the output of adrenal corticoids was studied. The parenteral administration of hypoglycin at 10 or 15 mg/100 g did not significantly change the adrenal corticoid secretion pattern. Also, when hypoglycin was incubated, at 200 μg to 2000 μg/ml of medium, with adrenal slices from untreated rats there was no statistically significant effect on corticoid output.

DISCUSSION

Hypoglycin apparently induces hypoglycemia in a unique manner. Although blood glucose levels are markedly decreased by 3 hr after hypoglycin treatment, little other effect on overall glucose metabolism is evident. Glucose conversion to respiratory CO_2 is unaffected and the lack of action of hypoglycin on the conversion of glucose to liver lipid or muscle glycogen has been previously reported.[8] The only direct effect on carbohydrate metabolism revealed from the present study is an inhibition of net glycogen synthesis from glucose in diaphragm. Although Patrick has reported an inhibition of glycogen formation from glucose in the liver,[7] interpretation of his results is difficult.

The dominant effects of hypoglycin are apparently those concerned with fatty acid metabolism. Following its administration *in vivo*, hypoglycin induces a pronounced increase in serum NEFA level, a moderate increase in the total lipids of the liver, and a significant inhibition of the rate of the conversion of butyrate or stearate to respiratory CO_2. Moreover, liver mitochondria isolated from hypoglycin-treated rats show an impaired ability to form high-energy phosphate bonds, an effect which has been reported to be correlated with the ability of a compound to induce fat infiltration in the liver.[21]

Hypoglycin does not immediately affect blood sugar levels as does insulin, but causes a profound lowering within 3 or 4 hr. This delayed effect may be because hypoglycin is metabolized to an active metabolite or possibly because the effects on blood sugar are secondary to some primary metabolic block. Both possibilities could occur. The fact that analogues without the double bond or the *cyclo*propane ring are not active as hypoglycemic agents suggests the uniqueness of this combination for hypoglycemic activity.[3]

The inhibition of oxidative phosphorylation observed in liver mitochondria of hypoglycin-treated rats and the lack of inhibition when hypoglycin is added *in vitro* to normal mitochondria further supports the idea that a metabolite of hypoglycin is the true hypoglycemic agent. Finally, methylene*cyclo*propanepyruvic acid and methylene*cyclo*propaneacetic acid, both likely metabolites, do produce hypoglycemia.[8] It is possible that such metabolites interfere with fatty acid oxidation either by binding coenzyme A or by interfering with the oxidation of normal fatty acid substrates in some other manner.

When administered *in vivo*, hypoglycin had no effect on the conversion of U-^{14}C-glucose or 1-^{14}C-acetate to respiratory CO_2, but it significantly inhibited the conversion of 1-^{14}C-labeled butyrate ,palmitate and stearate to CO_2. The total amount of respired

CO_2 was always reduced. These data support the idea of a metabolic block in the oxidation of fatty acids.* However, it must be pointed out that during the time interval of these studies a significant rise of serum NEFA was occurring.[8] This rise in NEFA may dilute the fatty acid pool sufficiently to account for the observed decrease in oxidation of the labeled acids.

Hypoglycin, 2-methylene*cyclo*propanepyruvic acid and 2-methylene*cyclo*propaneacetic acid had no effect on acetate metabolism in liver slices; 4-pentenoic acid, which inhibited the metabolism of acetate in the liver slice system, was only a weakly-active hypoglycemic agent in the intact animals.[23] The oxidation of butyrate by liver slices in the presence of hypoglycin or of the possible active metabolites of hypoglycin, such as 2-methylene*cyclo*propaneacetic acid, has not yet been studied.

SUMMARY

(1) Some effects of hypoglycin, α-aminomethylene*cyclo*propanepropionic acid, on certain aspects of glucose and fatty acid metabolism in the rat have been presented.

(2) Hypoglycin does not influence the conversion of glucose-U-^{14}C or acetate-1-^{14}C to respiratory CO_2 either in the intact rat or when added *in vitro* to normal liver slices.

(3) Hypoglycin markedly decreases the rate of conversion of butyrate-1-^{14}C or stearate-1-^{14}C to respiratory CO_2 in the intact rat.

(4) Liver mitochondria from hypoglycin-treated rats show an impaired ability to synthesize high-energy phosphate bonds associated with the oxidation of pyruvate and malate.

(5) The synthesis of glycogen from glucose in diaphragm is decreased by hypoglycin both when it is administered *in vivo* or added *in vitro*. Glucose uptake is unaltered in both cases.

(6) A small, but statistically significant decrease in R.Q. is observed in liver slices prepared from hypoglycin-treated rats.

(7) Hypoglycin is shown to have no effect on adrenal corticoid output when administered *in vivo* or added *in vitro* to normal adrenal slices.

(8) The evidence is discussed in connection with a possible inhibitory effect of hypoglycin on fatty acid metabolism.

Acknowledgement—The writers are indebted to Dr. P. H. Bell and to Dr. B. L. Hutchings for their interest in these studies.

* von Holt[22] has also recently suggested that hypoglycin may affect fatty acid oxidation.

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PREVENTION OF THE INHIBITORY EFFECTS OF URETHAN, FORMAMIDE, AND N-METHYLFORMAMIDE ON THE GROWTH OF *ESCHERICHIA COLI**

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Abstract—Purines, pyrimidines, ribonucleosides, ribonucleotides, carboxylic acids, and amino acids were tested for activity in preventing the growth-inhibition of *Escherichia coli* by urethan, formamide and N-methylformamide. 2 : 6-Diaminopurine and phenylalanine were the only compounds tested that caused true reversal of the inhibition of growth of *E. coli* 9637 by these agents, but neither of these compounds re-reversed the inhibition of a purine-requiring mutant or, in experiments with urethan, the inhibition of a pyrimidine-requiring mutant. On the other hand, glutamic acid caused true reversal of the inhibition of the purine-requiring mutant and, in experiments with urethan, of the inhibition of the pyrimidine-requiring mutant, but the apparent reversal of the inhibition of *E. coli* 9637 by glutamic acid was found to be due to stimulation of the overgrowth of drug-resistant mutants. A number of other compounds also stimulated the overgrowth of drug-resistant mutants in overnight growth experiments.

Urethan, formamide and N-methylformamide, individually, inhibited the formation of diazotizable amine by resting *E. coli* B96 cells. The inhibition by urethan was partially prevented by phenylalanine; the inhibition by formamide was partially prevented by glutamic acid and by 2 : 6-diaminopurine; and the inhibition by N-methylformamide was partially prevented by 2 : 6-diaminopurine.

The data indicate that there are probably multiple sites of action for each of these inhibitors.

INTRODUCTION

THE anti-leukemic action of urethan,[1] formamide,[2] and N-methylformamide[2] has been known for a number of years. Although the mutagenic, antimitotic, and carcinogenic effects of the agents and the activities of several compounds in preventing these effects point to an influence of each agent on the biosynthesis, metabolism, or funtioning of nucleic acids,[3–11] the mechanisms of action are still unknown. However, the results of an earlier study[11] did indicate that the mechanisms of inhibition by these compounds might be closely related. Therefore, the three were studied together in the present investigation, which was undertaken to obtain information on the sites of inhibition of the growth of *Escherichia coli*. The roles of these agents in inhibiting growth of bacteria may, of course, be different from their roles in influencing the metabolism of mammalian cells; however, it was hoped that the results of studies with bacteria might serve as a guide for studies with mammalian cells.

METHODS

The basal medium for the experiments with *E. coli* 9637, the drug-resistant mutants

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of *E. coli* 9637, and the purine-requiring mutant, *E. coli* B96, was that of Davis and Mingioli.[12] For the drug-resistant mutants this basal medium was supplemented with the respective inhibitor, and for the B-96 mutant the basal medium was supplemented with hypoxanthine at a level of 20 μg/ml. The pyrimidine-requiring mutant of *E. coli*, ATCC 9723 g, was grown on the basal medium of Yates and Pardee[13] supplemented with uracil at a concentration of 22 μg/ml. The inocula were prepared by adjusting a 24 hr culture to a transmission of 26 per cent using a Spectronic "20" colorimeter at 660 mμ and diluting the resulting solution tenfold with physiological saline; 0·1 ml of this dilute suspension was used to inoculate 10 ml of medium. Incubation was at 37° C, and percentage transmission was measured by means of a Bausch and Lomb Monochromatic Colorimeter using a 660 mμ interference filter or by means of a Bausch and Lomb Spectronic "20" colorimeter with a wavelength setting of 660 mμ.

Experiments related to the accumulation of 4-amino-5-imidazolecarboxamide by non-proliferating *E. coli* B96 cells were performed by a procedure essentially the same as that of Gots and Love.[14] Following growth in the presence of hypoxanthine, the cells were collected by centrifugation, washed three times with 0·067M phosphate buffer, pH7·2, and suspended in 0·067M phosphate buffer at pH7·2 to which glucose, 360 μg/ml, and the test compounds had been added. After incubation of the suspension for 16 hr, the metabolic reactions of the non-proliferating cells were terminated by the addition of trichloroacetic acid, and the concentration of diazotizable amine was determined by a modification of the method of Bratton and Marshall.[15]

In some experiments, the test compounds were added when the bacteria were in the exponential phase of growth, and the subsequent rate of multiplication was followed turbidimetrically. For the inoculum in these experiments, the cells from 20 ml of a 24 hr culture of the bacteria were collected by centrifugation, washed once with saline, and suspended in 3 ml of saline. A sufficient volume of this suspension was added to 10 ml of medium in a 300 ml Erlenmeyer flask to give a transmission of 65–70 per cent. Determination of transmission at various periods was facilitated by fitting the flask with a standard culture tube by means of a standard taper ground glass joint; the contents of the flask were transferred to the tube by inverting the flask. The flask and contents were incubated, and the turbidity was determined at intervals of 15 or 30 min. Soon after the rate of growth had entered the exponential phase, the inhibitor and candidate reversal agents were added to the flasks, and periodic determinations of turbidity were continued. The growing cultures were not agitated or continually aerated while they were in the incubator, but the relatively large surface area of the medium in the flask and the necessary inversion of the flask for determination of turbidity caused considerable aeration.

In some of the experiments it was desirable to determine if the cells that eventually grew in the inhibited culture and the cells that grew in the presence of the inhibitor plus the test compound were less sensitive to the inhibitor than the initial culture. For these determinations, the cells were washed with saline and used to inoculate tubes of medium containing various concentrations of inhibitor. The extent of growth was measured after overnight incubation.

RESULTS

The data of Table 1 show the degree of inhibition of the growth of *E. coli* 9637 by rather large quantities of formamide, N-methylformamide or urethan and the extent

TABL I. GROWTH OF *E. coli* 9637 IN THE PRESENCE OF INHIBITORS AND GLUTAMIC ACID, PHENYLALANINE, OR 2 : 6-DIAMINOPURINE

Test compound	Conc. (μmoles/ml)	Growth, % of control			
		Formamide*	N-Methyl-formamide†	Urethan‡	
				Expt. 1	Expt. 2
None	—	6	8	2	2
Glutamic acid	5	106 (A)§	54 (D)	118	—
	15	—	—	—	110 (G)
Phenylalanine	5	94 (B)	62 (E)	20	54 (H)
2:6-Diaminopurine	0·033	79 (C)	51 (F)	24	—
	0·1	77	38	43	—
	0·2	62	33	48	—

* Formamide was present at a concentration of 17·5 mg/ml.
† N-Methylformamide was present at a concentration of 20 mg/ml.
‡ Urethan was present at a concentration of 10 mg/ml.
§ The letters in parentheses are used to indicate which cultures were used for inocula in the tests of resistance reported in Table 2.

of growth that occurred when glutamic acid, phenylalanine, or 2 : 6-diaminopurine was present in addition to the inhibitor. The data of Table 2, however, show that the cells which grew in the presence of inhibitor and glutamic acid were somewhat resistant to the inhibitor, while those which grew in the presence of inhibitor and

TABLE 2. TESTING OF CULTURES OF TABLE 1 FOR DRUG RESISTANCE

Inhibitor	Concentration (mg/ml)	Growth, % of controls			
		Fresh culture	A*	B	C
Formamide	0	100	100	100	100
	12·5	13	87	11	12
	15·0	3	87	2	3
	17·5	0	76	0	1
	20·0	0	66	0	0
		Fresh culture	D	E	F
N-Methylformamide	0	100	100	100	100
	17·5	4	45	2	4
	20·0	1	36	0	1
	22·5	0	26	0	0
	25·0	0	19	0	0
		Fresh culture	G	H	
Urethan	0	100	100	100	
	7·5	1	91	1	
	10·0	0	64	0	
	12·5	0	34	0	
	15·0	0	12	0	
	17·5	0	3	0	
	20·0	0	0	0	

* The letters indicate the cultures of Table 1 that were used as inocula.

phenylalanine or 2 : 6-diaminopurine were as sensitive to the inhibitor as cells that had never been exposed to the inhibitor. The possibility that the growth was due to the presence of glutamic acid that was carried along with the cells of the inoculum seems unlikely because of the small size of the inoculum. Also, the cells that grew in the presence of inhibitor and glutamic acid were about equally as resistant to the inhibitor as cells that were exposed to the inhibitor alone and which proliferated during a long period of incubation. These results are interpreted to indicate that glutamic acid stimulated the overgrowth of resistant cells, while phenylalanine and 2 : 6-diaminopurine caused true prevention of the inhibition. In Table 3 data are given which show

TABLE 3. GROWTH OF *E. coli* 9637 IN THE PRESENCE OF INHIBITORS AND COMBINATIONS OF PHENYLALANINE AND GLUTAMIC ACID

Inhibitor	Concentration (mg/ml)	Growth,* % of controls reversal agent			
		No reversal agent	Phenylalanine†	Glutamic acid‡	Phenylalanine† plus glutamic acid‡
Formamide	0	100	98	94	94
	15·0	24	94	53	77
	17·5	12	91	48	75
	20·0	4	54	37	82
N-Methylformamide	0	100	98	90	90
	17·5	17	64	17	43
	20·0	8	59	9	31
	22·5	5	45	11	18
Urethan	0	100	95	90	—
	10·0	2	4	79	59
	12·5	2	1	29	14
	15·0	1	0	13	1

* Growth was measured turbidimetrically after incubation at 37 °C for 16 hr.
† Phenylalanine was used at a concentration of 7·6 μmoles/ml.
‡ Glutamic acid was used at a concentration of 10 μmoles/ml.

the reversal of growth inhibition by phenylalanine and glutamic acid, as added to the medium singly and in combination, with various concentrations of the inhibitors. Cells that grew in the presence of both of these compounds retained sensitivity to the inhibitors.

A number of other compounds in quantities that were equimolar to glutamic acid were tested for prevention of inhibition by each of the three inhibitors, and, when growth occurred, the cells were tested for resistance. The results are indicated in Table 4. Glutamic acid stimulated the overgrowth of resistant cells much more than any other compound, and none of these other compounds caused true prevention of inhibition.

Fig. 1 shows that *E. coli* 9637 is inhibited by urethan when the inhibitor is added to the culture during the exponential phase of growth and that the inhibition is partially prevented by phenylalanine. This figure also shows that the inhibition is partially overcome even if the addition of the phenylalanine is delayed until 1 hr or longer after the addition of the inhibitor. Under the same conditions glutamic acid did not prevent or overcome the inhibition, even though it was added simultaneously

TABLE 4. EFFECTS OF VARIOUS COMPOUNDS UPON THE GROWTH OF *E. coli* 9637 IN THE PRESENCE OF FORMAMIDE, N-METHYLFORMAMIDE, OR URETHAN

No effect		Stimulation of overgrowth of drug-resistant cells	True reversal
Adenosine	Glycine	Ureidosuccinic acid	Phenylalanine
Guanosine	Alanine	Orotic acid	2:6-Diaminopurine
Uridine	Serine	Uracil	
Cytidine	Threonine	Cytosine	
Adenylic acid (2′ + 3′)	Leucine	Thymine	
	Lysine	Thymidine	
Uridylic acid (2′ + 3′)	Ornithine	Cytidylic acid (2′ + 3′)	
	Citrulline	Glutamic acid	
	Arginine	Aspartic acid	
Citric acid	Histidine	Asparagine	
*iso*Citric acid	Proline	Methionine	
*cis*Aconitic acid	Hydroxyproline	Valine	
Succinic acid	Cystine	*iso*Leucine	
Fumaric acid	Tyrosine		
Malic acid	Tryptophan		
Oxalacetic acid			
α-Ketoglutaric acid			

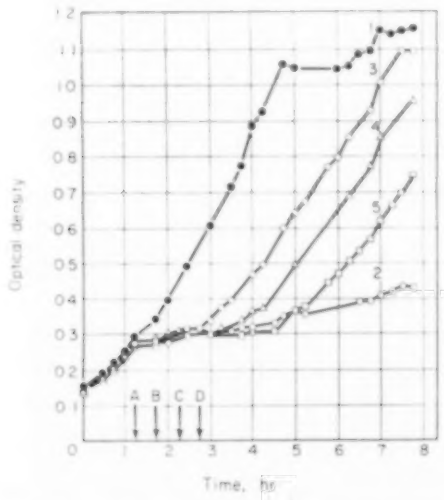

FIG. 1. Reversal of urethan inhibition of *E. coli* 9637 by phenylalanine. Culture (1) is the control. To cultures (2), (3), (4) and (5) urethan was added at time *A* to give a concentration of 10 mg/ml, and to cultures (3) (4) and (5) phenylalanine was added at times *B*, *C* and *D*, respectively, to give a concentration of 0·81 mg/ml.

with the inhibitor. Similar results were obtained with formamide and N methylformamide. Evidently, no significant overgrowth of resistant cells occurred in the presence of glutamic acid under these conditions and during this relatively short period of growth.

In order to obtain information regarding the correlation of molecular structure and activity in preventing inhibition, several purines and related compounds were

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tested with exponentially growing cultures, and some of the results are shown in Fig. 2. For comparison, phenylalanine was included in this experiment. 2 : 6-Diaminopurine was the most active of the purines, and 2-aminopurine had considerable activity, but 2-hydroxypurine was inactive. In other experiments not shown here, 2 : 6-diamino-8-azapurine possibly had slight activity, but 4-amino-5-imidazolecarboxamide, hypoxanthine, inosine, *iso*guanine, 2-hydroxypurine, guanine, xanthine, adenine and 8-azaguanine had little or no activity. It was also found that 2 : 6-diaminopurine could overcome the effects of urethan when the reversal agent was added to cultures that were already inhibited by urethan.

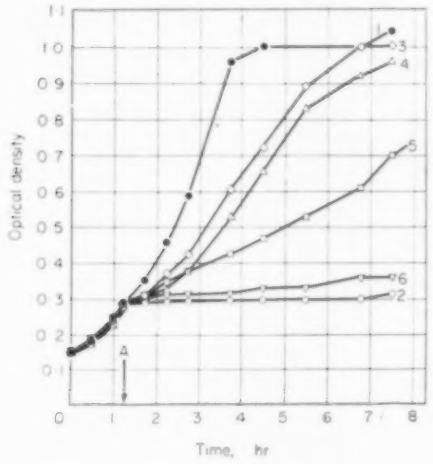

FIG. 2 Prevention of urethan inhibition of *E. coli* 9637 by phenylalanine and by purines. Culture (1) is the control. To cultures (2), (3), (4), (5) and (6) urethan was added at time *A* to give a concentration of 10 mg/ml. To cultures (3), (4), (5) and (6) the test compounds were added at time *A* to give the indicated concentrations: culture (3)—phenylalanine, 0·81 mg/ml; culture (4)—2:6-diaminopurine, 0·1 mg/ml; culture (5)—2-aminopurine, 0·09 mg/ml; culture (6)—2-hydroxypurine, 0·09 mg/ml.

It was desirable to determine whether a culture that had been inhibited by formamide, N-methylformamide, or urethan after it had entered the exponential phase of growth would resume exponential growth if the inhibitor was removed. Accordingly, the following experiments were performed. After the cultures were well into the exponential phase of growth, the inhibitors were added, and the cultures were exposed to the inhibitors for 2 hr, during which time essentially no growth occurred. At the end of the 2 hr, the control cultures and the inhibited cultures were centrifuged, and the cells were washed once with saline and resuspended in fresh medium that contained no inhibitor. The process of collecting cells, washing them, and resuspending them required about 60 min, and since some cells were lost by decantation, the optical densities of the reconstituted cell suspensions were less than those of the original cultures prior to centrifugation. Upon incubation of the suspensions, the control cultures immediately resumed exponential growth, and after an initial lag phase the inhibited cultures also grew exponentially.

Table 5 contains data obtained in tests of cross-resistance with various mutant lines of *E. coli*. The azaserine-resistant mutant was somewhat resistant to formamide, N-methylformamide, and urethan. The DON-resistant mutant was also somewhat

TABLE 5. TESTS OF CROSS-RESISTANCE

Lines of *E. coli*	Concentration (mg/ml) of inhibitor required to cause 50% inhibition of growth				
	Azaserine	DON*	Formamide	N-Methyl-formamide	Urethan
9637	0·0045	0·000053	7·9	8·3	10·5
Azaserine-resistant	>0·2†	0·083	27·7	16·3	18·0
DON-resistant	0·00057	0·073	26·6	20·2	NT‡
Formamide-resistant	0·000053	0·00005	24·3	18·3	NT
N-Methylformamide-resistant	0·000052	0·00005	27·9	25·0	NT
Urethan-resistant	0·000051	0·00005	NT	NT	22·9

* 6-Diazo-5-oxo-L-norleucine.
† Azaserine at a concentration of 0·2 mg per ml caused only 6% inhibition of growth.
‡ NT indicates that no test was performed.

resistant to formamide and N-methylformamide; this mutant was not tested for resistance to urethan. However, it is of interest that the formamide-resistant mutant, the N-methylformamide-resistant mutant, and the urethan-resistant mutant showed mutual cross-resistance to formamide, N-methylformamide, and urethan as previously reported,[11] yet retained their sensitivity to azaserine and DON.

Table 6 contains data that show the extent of growth of *E. coli* B96 cells in the

TABLE 6. GROWTH OF *E. coli* B96 IN THE PRESENCE OF INHIBITORS AND GLUTAMIC ACID, GLUTAMINE, PHENYLALANINE, OR 2 : 6-DIAMINOPURINE

Test compound	Concentration (μmoles/ml)	Growth, % of control		
		Formamide*	N-Methylformamide*	Urethan†
None	—	2	8	2
Glutamic acid	5	64	20	78
	10	66	15	76
Glutamine	5	2	6	8
	10	1	5	10
Phenylalanine	5	2	5	0
	10	2	5	0
2:6-Diaminopurine	0·33	1	5	7
	0·66	—	—	8
	1	2	4	—
	2·64	—	—	0

* The inhibitor was present at a concentration of 30 mg/ml, and the turbidity was determined after incubation at 37 °C for 16 hr.
† Urethan was present at a concentration of 10 mg/ml, and the turbidity was determined after incubation at 37 °C for 16 hr.

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presence of the inhibitors and glutamic acid, glutamine, phenylalanine, or 2 : 6-diaminopurine. Extensive growth occurred in the presence of the inhibitors only when glutamic acid was present, and the results of tests for resistance showed that this growth was not due to the overgrowth of resistant cells. Formamide slowed the growth of exponentially growing B96 cells, and N-methylformamide stopped the growth of such cells. The quantities of inhibitor required to inhibit the growth of cells under these conditions were considerably greater than those required to inhibit the growth of the 9637 cells under similar conditions and also greater than the quantities required to inhibit the growth of B96 cells in overnight growth studies. Glutamic acid partially prevented the inhibition by each of these agents. As shown in Fig. 3, the inhibition of B96 cells by N-methylformamide was partially overcome by glutamic acid even if the inhibitor was added two hours before the addition of the glutamic acid.

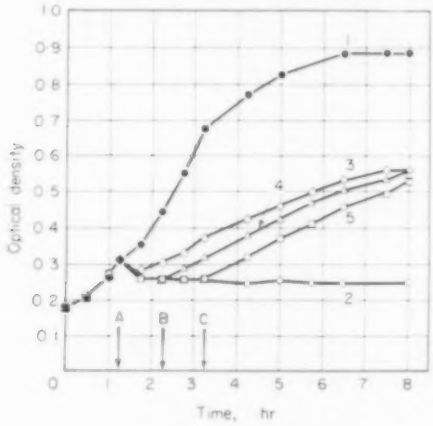

FIG. 3. Reversal of N-methylformamide inhibition of *E. coli* B96 by glutamic acid. Culture (1) is the control. To cultures (2), (3), (4) and (5) N-methylformamide was added at time *A* to give a concentration of 50 mg/ml, and to cultures (3), (4) and (5) glutamic acid was added at times *A*, *B* and *C*, respectively, to give a concentration of 2·19 mg/ml.

The data of Table 7 show that formamide, N-methylformamide, and urethan partially inhibit the formation of diazotizable amine (4-amino-5-imidazolecarboxamide) by resting B96 cells. The inhibition by formamide was prevented to a greater extent by glutamic acid than by 2 : 6-diaminopurine or phenylalanine, although these compounds also were somewhat effective. The inhibition by N-methylformamide was partially prevented by 2 : 6-diaminopurine but not by glutamic acid or phenylalanine. The inhibition by urethan was partially prevented by phenylalanine but not by glutamic acid or 2 : 6-diaminopurine. These results indicate a certain degree of specificity of the inhibitors.

A number of compounds including phenylalanine and glutamic acid were tested for the prevention of the inhibition of growth of the pyrimidine-requiring mutant 9723 g by urethan. Glutamic acid was the only compound that was found to give true prevention of inhibition (Table 8); 2 : 6-diaminopurine was not tested. No similar tests were performed with formamide or N-methylformamide.

TABLE 7. EVALUATION OF COMPOUNDS FOR PREVENTING THE INHIBITION OF THE PRODUCTION OF DIAZOTIZABLE AMINE BY *E. coli* B96

Test compound	Concentration (μmoles/ml)	Production of diazotizable amine (% of control)		
		Formamide* 35 mg/ml	N-Methylformamide† 20 mg/ml	Urethan† 30 mg/ml
None	0	49	33	47
Glutamic acid	5	64	31	50
Phenylalanine	10	55	32	80
2:6-Diaminopurine	0·05	59	53	47

* The recorded values are the average values for nine experiments. Although the order of the activities was not consistent for all of the experiments, glutamic acid was the most active compound in six of the experiments.

† The recorded values are the average values for four experiments, and the order of the activities of the compounds was the same for all of the experiments.

TABLE 8. GROWTH OF *E. coli* 9723 g IN THE PRESENCE OF URETHAN PLUS GLUTAMIC ACID AND TESTING OF THE RESULTING CULTURE FOR RESISTANCE TO URETHAN

A. Growth

Tube	Urethan (mg/ml)	Glutamic acid (μmoles/ml)	% growth, after 16½ hr.
1	0	0	100
2	15	0	2
3	15	5	78

B. Test for Resistance

Urethan (mg/ml)	% growth	
	Culture from tube 1*	Culture from tube 3*
0	100	100
10·0	36	22
12·5	2	1
15·0	1	0
17·5	0	0
20·0	0	0

* This number indicates the tube of *A* that was used as inoculum in the test for resistance.

DISCUSSION

The activity of phenylalanine in preventing the inhibition of the wild strain of *E. coli* by formamide, N-methylformamide, and urethan is in contrast to the lack of activity of tyrosine and tryptophan and to the failure of phenylalanine to prevent the inhibition of the growth of the purine-requiring mutant and of the pyrimidine-requiring mutant. The various roles of phenylalanine in the metabolism of *E. coli* are not known at present. It is known that phenylalanine will prevent the inhibition

of the growth of *E. coli* by azaserine,[16, 17] but the mechanism of this prevention is not established. Phenylalanine is one of several compounds that prevented the azaserine inhibition of the production of diazotizable amine, an intermediate in the biosynthesis of purines, by resting B96 cells, [17–19] and, in the presently reported experiment, it partially prevented the urethan inhibition and formamide inhibition of the production of diazotizable amine. These results imply that phenylalanine may be involved in the biosynthesis of purines, but this evidently is not its only role, because it is more effective than intact purines in preventing the inhibition by azaserine, formamide, N-methylformamide, and urethan. The possibility that phenylalanine exerts its effect by preventing the entry of the inhibitor into the cell[20] has not been eliminated, but the growth of the cells can be arrested for an hour or more prior to addition of the phenylalanine, and growth will resume soon after the addition. Since phenylalanine does not prevent inhibition of the purine-requiring and pyrimidine-requiring cells, phenylalanine must not perform as important a function in these mutant lines as it does in the wild strain, or supplementation by phenylalanine alone is not sufficient to overcome the inhibition by the higher concentration of inhibitor. Phenylalanine also did not prevent inhibition of *E. coli* 9637 at higher concentrations of inhibitor. This might indicate that the inhibitor prevents the utilization of phenylalanine, rather than its synthesis, or it may indicate that there are several sites of action and that at the higher concentration the inhibitor becomes effective at additional sites.

2 : 6-Diaminopurine was the most active, on a molar basis, of the compounds that were tested as reversal agents for these inhibitors. However, the effectiveness of 2 : 6-diaminopurine is limited because this compound itself is toxic at higher concentrations. Comparison of the activities of the various substituted purines show that there is some specificity for an amino group on carbon-2. 2-Aminopurine showed some activity, but adenine was inactive. Guanine, *iso*guanine, 2-hydroxypurine, xanthine and hypoxanthine were also inactive. Therefore, it appears that an amino group on carbon-2 is essential for activity, but the activity is greater if there is also an amino group on carbon-6. A hydroxyl group on carbon-6 eliminates any activity that might be conferred by the 2-amino group. Replacement of carbon-8 of 2 : 6-diaminopurine by a nitrogen atom results in a great decrease of activity. 2 : 6-Diaminopurine has an activity qualitatively similar to that of phenylalanine, in that it prevented inhibition of *E. coli* 9637 without stimulating the overgrowth of resistant cells, and it did not prevent inhibition of the B96 mutant. Like phenylalanine, 2 : 6-diaminopurine partially prevented the inhibition of formation of diazotizable amine by formamide, and, unlike phenylalanine, it also partially prevented the inhibition by N-methylformamide.

Table 9 shows a comparison of the effects of urethan, formamide, N-methylformamide, azaserine and DON upon certain responses of bacteria and of resistant lines of the plasma-cell neoplasm 70429. This table contains data that were taken from the literature as well as those obtained in the present study. This comparison shows that the five inhibitors have certain effects in common, but each inhibitor has certain effects that are peculiar to that particular inhibitor.

The inhibitory action of formamide, N-methylformamide, and urethan may occur within the cell or at the cell wall.[20, 21] The inhibitory effect is transient, and growth resumes if the inhibited cells are transferred to fresh medium that does not contain the inhibitor. If inhibition is dependent upon the continual entrance of the inhibitor into the cell, then the reversal agent might function by competing with the inhibitor

for the transport system.[20] If inhibition is dependent simply upon the presence of the inhibitor within the cell, then the reversal agent is perhaps competing with the inhibitor for an enzyme. Another possibility is that the reversal agent effectively detoxifies the inhibitor by chemically reacting with it, but this mechanism seems unlikely in view of the known chemical properties of these compounds.

TABLE 9. COMPARISON OF EFFECTS OF INHIBITORS

Property	Inhibitor				
	Azaserine	DON	Urethan	Formamide	N-Methyl-formamide
Resistance of azaserine-resistant mutant of *E. coli*.	+	+	+	+	+
Resistance of DON-resistant mutant of *E. coli*.	—	+	NT *	+	+
Resistance of urethan-resistant mutant of *E. coli*.	—	—	+ [11]	± [11]	± [11]
Resistance of formamide-resistant mutant of *E. coli*.	—	—	± [11]	+	+
Resistance of N-methylformamide-resistant mutant of *E. coli*.	—	—	± [11]	+	+
Resistance of azaserine-resistant line of neoplasm 70429.	± [21]	± [21]	NT	NT	± [21]
Resistance of DON-resistant line of neoplasm 70429.	± [21]	± [21]	NT	NT	± [21]
Resistance of N-methylformamide-resistant line of neoplasm 70429.	± [21]	± [21]	NT	NT	± [21]
Effectiveness of phenylalanine in preventing inhibition of growth of *E. Coli*.	± [18]	—	+	+	+
Effectiveness of 2 : 6-diaminopurine in preventing inhibition of growth of *E. coli*.	NT	NT	+	+	+
Effectiveness of purines other than 2 : 6-diaminopurine in preventing inhibition of growth of *E. coli*.	± [22]	± [22]	—	—	—
Inhibition of *de novo* biosynthesis of purines.	± [22]	± [22]	± [3, 5, 7, 8]	NT	± [5], + [3]
Inhibition of formation of diazotizable amine by *E. coli*. B96.	± [14]	NT	+	+	—
Reversal of inhibition of formation of diazotizable amine by phenylalanine.	± [14]	NT	+	±	—
Reversal of inhibition of formation of diazotizable amine by 2 : 6-diaminopurine.	NT	NT	—	±	—
Reversal of inhibition of formation of diazotizable amine by glutamic acid.	+ [14]	NT	—	+	—
Accumulation of 1-(N-formylglycinamide) ribose by inhibited *E. coli*.	± [24]	± [23]	± [25]	± [25]	± [25]

* NT = not tested.

The data that are presented in this report suggest that in *E. coli* there are multiple sites of action for each of these inhibitors—formamide, N-methylformamide, and urethan. Possible sites might be represented schematically as follows:

(1) A → B, reversed by phenylalanine and 2 : 6-diaminopurine.

(2) C → D, reversed by glutamic acid.

Each of these steps might be inhibited by each of the agents, but process (1) is more sensitive to the agents, and hence the effects of low levels of the inhibitors can be overcome by phenylalanine or 2 : 6-diaminopurine. At these low concentrations of inhibitor, process (2) is not affected. However, at higher levels both processes are affected, and phenylalanine or 2 : 6-diaminopurine does not prevent the inhibition. If process (1) is sufficiently less critical in the purine-requiring cells and the pyrimidine-requiring cells than in the wild-type cells, then these auxotrophic cells would not be sensitive to low levels of the inhibitors, but at higher levels they would be inhibited because of interference with process (2). This would explain why larger quantities of the agents would be required to inhibit the mutant cells. Since the characteristic features of these mutants are the lack of purine synthesis and the lack of pyrimidine synthesis, it might be suspected that process (1) is related directly or indirectly to the synthesis of purines and pyrimidines. This possibility is consistent with the observed interference with the synthesis of diazotizable amine. Perhaps both process (1) and process (2) actually consist of several different biochemical transformations, and this

accounts for the observed differences with the individual inhibitors. This possibility is consistent with the fact that combinations of phenylalanine and glutamic acid do not completely prevent inhibition. Present knowledge does not permit further definition of processes (1) and (2), but it is hoped that experiments in progress with radioactive substrates will elucidate them.

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EFFECT OF RESERPINE, SEROTONIN, AND 5-HYDROXYTRYPTOPHAN ON RABBIT PLATELET HISTAMINE *IN VIVO* AND *IN VITRO**

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(*Received* 26 *February* 1960)

Abstract—Reserpine, which releases serotonin (HT) and catecholamines from tissues, does not release histamine from various tissues of the rabbit *in vivo*, with the exception of platelets. HT and its biologic precursor, 5-hydroxytryptophan (5HTP), also effect the release of platelet histamine *in vivo*. Neither reserpine nor HT releases platelet histamine *in vitro*.

IT HAS been amply demonstrated that reserpine and other centrally active Rauwolfia alkaloids effect the release of serotonin (HT) and catecholamines from various organs of several animal species.[1,2] Administration of reserpine to rabbits also causes a decline in the histamine levels in blood[3] where most of the histamine content is localized in the platelets.[4] This finding contrasts with the observation in this laboratory that reserpine releases HT but not histamine from rabbit platelets *in vitro*.[5,6]

By taking advantage of the sensitivity of a recently developed fluorometric method for histamine assay, we have been able to show in the present studies that reserpine does not release histamine *in vivo* from various rabbit organs other than platelets. The studies further demonstrate that HT and its precursor, 5-hydroxytryptophan (5HTP), as well as reserpine, cause a decline in the histamine levels of rabbit blood *in vivo*; the results suggest that HT may be implicated in the mechanism of histamine release by reserpine *in vivo*. However, none of these substances releases histamine from rabbit platelets *in vitro*.

METHODS

Histamine was measured by the fluorometric method of Shore *et al.*[7] Serotonin was estimated by the fluorometric method of Bogdanski *et al.*[8] after precipitation of proteins as described by Waalkes and Weissbach.[3] Estimation of the serotonin content of isolated platelets was carried out by the method described by Udenfriend *et al.*[9]

Adult male rabbits were given the various drugs by subcutaneous or intravenous injection or by slow intravenous infusion (0·2 ml/min) into an ear vein.

Studies on the release of serotonin and histamine from rabbit platelets *in vitro* were carried out as described by Carlsson *et al.*[5] In this procedure the extent of release is determined by measurement of amine levels in the supernatant fraction of plasma after removal of platelets by centrifugation.

* Preliminary accounts of portions of this work were presented before the Society for Pharmacology and Experimental Therapeutics at Atlantic City, N.J., April 1959, and Miami, Fla., September 1959.

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RESULTS

Effect of reserpine, HT and 5HTP on tissue histamine levels in vivo

Analysis of brain, lung, liver, heart and kidney of rabbits given reserpine 16 hr previously failed to show a release of histamine from these tissues (Table 1). However,

TABLE 1. RABBIT TISSUE HISTAMINE LEVELS IN NORMAL RABBITS AND RABBITS GIVEN RESERPINE (2 MG/KG) 16 HR PREVIOUSLY

Tissue	Histamine concentration (μg/g)*	
	Normal	Reserpine treated
Brain	0·20–0·40	0·22–0·37
Lung	1·9 –3·1	1·4 –2·8
Liver	0·53–1·1	0·51–0·93
Heart	0·45–1·2	0·49–1·1
Kidney	0·48–0·90	0·40–0·93

*Each range represents from three to five experiments.

TABLE 2. EFFECT OF RESERPINE AND METHYL RESERPATE ON HISTAMINE CONTENT OF RABBIT BLOOD *in vivo*

Alkaloids were dissolved in a solvent mixture of acetic acid, ethanol, propylene glycol and water[9] as described previously.[10] Control blood samples were taken and the drugs (2 mg/kg) were injected intravenously. Blood samples were taken 16 hr later. Values represent results of individual experiments.

Treatment	Decrease (%)
Reserpine	70, 67, 75
Methyl reserpate	11, 21, 15
Solvent	12 ,6, 10

in confirmation of the findings of Waalkes and Weissbach,[3] there was a marked decline in the levels of blood histamine (Table 2). Administration of the weakly sedative Rauwolfia alkaloid, methyl reserpate, caused a decline in blood histamine levels only slightly greater than observed in rabbits treated with the alkaloid solvent alone (Table 2). Intravenous infusion or injection of HT or 5HTP caused a marked decline in the blood levels of histamine (Table 3).

Since reserpine does not cause a decrease in the concentration of circulating platelets,[10] the decline in histamine levels following reserpine administration represents a true release from these cells. HT and 5HTP administration also cause a release from platelets *in vivo*, as shown by analysis of platelets taken from these rabbits (Table 3).

Effect of reserpine, HT and 5HTP on platelet histamine in vitro

Addition of reserpine to platelet-rich plasma resulted in a marked release of serotonin from the platelets, as reported previously,[5] but no significant release of histamine could be detected (Table 4). Furthermore, no histamine release could be detected

TABLE 3. EFFECT OF HT AND 5HTP ON RABBIT BLOOD HISTAMINE *in vivo*

Treatment	Decrease in Histamine Level, per cent	
	Whole Blood	Platelets
Infusion*		
HT, 4 μg/kg per min	38, 58, 48	18, 20
HT, 8 μg/kg per min	—	32, 28
Saline	18	0, 2
Injection		
HT†	—	26
5HTP‡	*27, 33*, 37, 45	*17, 31*, 26, 38
5HTP§	—	52, 59
Saline	2, 9, 5	0, 2

* Infusions into an ear vein were carried out for 4 hr at the rate of 0·2 ml/min. All values represent decrease from levels in same animals before treatment.

† HT, 1 mg/kg, was injected subcutaneously every hr for 4 hr. Blood was analyzed 1 hr after the final injection.

‡ 5HTP, 50 mg/kg, was injected subcutaneously. Blood samples were taken after 2 hr (italics) and 4 hr (roman).

§ 5HTP, 100 mg/kg, was injected in two divided doses 4 hr apart. Samples were taken 22 hr after the first injection.

TABLE 4. RELEASE OF HT AND HISTAMINE FROM RABBIT PLATELETS *in vitro*

(Platelet-rich plasma was incubated with the various agents at 37 °C under nitrogen. Procedure was that described by CARLSSON *et al.*[5] Each value represents a single experiment.)

Treatment	Amine released %			
	HT		Histamine	
	2 hr	4 hr	2 hr	4 hr
Reserpine 1 to 3 μg/ml	18, 20, 22	47, 46	0, 3, 0	6, 1
HT 2 μg/ml	—	—	0	2
HT 40 μg/ml	—	—	4, 2	—
5 HTP 2 μg/ml	—	—	0, 2	—

following incubation of platelets with HT or 5HTP. Control studies in which histamine was added to platelet-rich plasma showed that the added histamine remained in the supernatant fraction after removal of the platelets by centrifugation.

DISCUSSION

The observation that reserpine causes a decline in the level of histamine in rabbit blood suggests that the alkaloid might release histamine from many tissues in the same manner that it releases HT and catecholamines from their storage depots. Our findings show, however, that reserpine does not decrease the histamine levels of rabbit tissues other than blood.

A marked release of HT from tissues occurs following administration of reserpine but not methyl reserpate.[1,5] Since the same pattern is seen in the case of blood histamine release *in vivo*, it might appear that the mechanisms of release of the two amines from blood by reserpine are identical. However, while reserpine releases histamine from

platelets *in vivo*, it does not do so *in vitro* under conditions in which HT is readily released.

The restriction of the release of histamine *in vivo* to blood led us to consider that the HT released by reserpine might in turn act directly on the platelets to release histamine. There is some evidence that this series of events might occur since administration of HT or its precursor, 5HTP, caused a release of platelet histamine. Attempts to effect a release of platelet histamine *in vitro* by incubating platelets with HT or 5HTP were unsuccessful. Thus, reserpine and HT show a common pattern in their effects on platelet histamine.

At least two explanations exist which would be consistent with the difference between the results obtained *in vivo* and *in vitro*. One possible explanation is that the action of the various releasing agents *in vivo* is by a direct effect on platelets, which cannot be demonstrated *in vitro* because of the presence of the calcium-chelating agent, ethylenediaminetetra-acetate (EDTA), necessary for stabilization of isolated platelets.[11] There is some precedent for this possibility as Woolley has shown that removal of calcium ions by EDTA abolishes the uterus-contracting activity of HT *in vitro*.[12] This explanation would suggest that an interaction between calcium ions and HT is necessary for certain actions of HT, including histamine release from platelets.

Another possible explanation is that reserpine, HT and 5HTP exert a common action *in vivo* which indirectly causes a release of platelet histamine. Attempts to demonstrate this effect in a simple model system *in vitro* might then be impossible.

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SHORT COMMUNICATIONS

Notes on the reduction of biological acetylations with A-methopterine

(*Received* 15 *July* 1959)

In a recent publication[1], some interesting effects produced by A-methopterine on biological acetylation processes were reported. This folic acid analogue inhibited the acetylation of sulfanilamides and isoniazides in pigeon liver extracts, and lowered considerably the acetylated sulfanilamide blood levels of rabbits.

This report describes the effects produced by prolonged treatment with A-methopterine on the concentration in liver and the cellular distribution of the prosthetic group of the acetylating enzyme.

The experiments were performed on 20 young rats of our stock having an average weight of 120–150 g, and fed on a semisynthetic diet of a known composition, which contained 2 mg/kg folic acid (i.e. a quantity far in excess of normal requirements). A water suspension of A-methopterine (4-amino-N_{10}-methyl-pteroilglutamic-acid) was administered orally with the aid of a small esophageal probe in doses of 30 mg/kg.

Treatment was carried on for 7 days; 24 hr after the administration of the last dose, the animals were bled to death. Their livers were aseptically removed, cleansed in physiological solution, weighed and divided into two sections of known weight; the smaller section was employed to determine the concentration of co-enzyme A and total proteins; the larger one was centrifuged in accordance with standard procedure for the separation of nuclei (at 500 r.p.m.), mitochondria (at 3000 r.p.m.) and microsomes (at 20,000 rev/min).

The co-enzyme A contents was determined by Kaplan and Lipmann's method[2], while proteins were assessed by the method employed by Lowry *et al.*[3]. Results are shown in Fig. 1.

Table 1. Distribution of co-enzyme A in the different fractions of liver homogenate taken from animals treated with A-methopterine

Animal group	Homogenate fraction	Co-enzyme A	
		μ/g liver	μ/100 mg/proteins
Controls (8)*	Total homogenate	154·2 ± 6·1	85·6 ± 4·1
	Mitochondria	85·0 ± 4·8	157·6 ± 8·8
	Nuclei	29·7 ± 1·5	92·8 ± 3·2
	Microsomes	5·4 ± 0·2	26·7 ± 1·5
	Supernatant	33·1 ± 1·8	47·2 ± 2·2
Treated (10)*	Total homogenate	62·9 ± 5·5	41·8 ± 3·7
	Mitochondria	19·5 ± 1·2	38·2 ± 3·0
	Nuclei	20·8 ± 1·6	86·5 ± 7·4
	Microsomes	2·9 ± 0·2	24·1 ± 2·3
	Supernatant	19·4 ± 1·4	32·1 ± 2·9

* No. of animals in each group.

It may be noted that co-enzyme concentration fell by 59 per cent when based on weight and by 51 per cent when based on protein concentration; it would therefore seem clear that diminished co-enzyme A activity cannot be considered a result of a possible cellular fibrosis or necrosis. The mitochondrial fraction of co-enzyme A was most affected by A-methopterine treatment, its concentrations falling to values 77 per cent below normal. A lesser reduction was noted in co-enzyme A contents of the nuclei, microsomes and supernatant bodies; in all of these there was a considerable

fall in the protein content suggesting that widespread liver damage caused by A-methopterine. Although the methods employed in these investigations could not determine whether A-methopterine exerts its action by blocking the biosynthesis of co-enzyme A, or by inhibiting this latter's acetylating effect, it would appear likely that, given the doses used, both mechanisms were involved to a substantial degree. This hypothesis would seem to be supported by the fact that, in animals that were treated with either panthotheine (an intermediate product in the biosynthesis of co-enzyme A) or with adenosintriphosphate, the effect of A-methopterine on the acetylating processes was much smaller.[4]

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The effect of disulfiram on the metabolism of normetanephrine-1-^{14}C in the guinea pig

(*Received* 14 *April* 1960)

THE oxidation of acetaldehyde is inhibited, both *in vivo* and *in vitro* by disulfiram*, a drug used in the treatment of alcoholism.[1] The oxidation of β-substituted acetaldehydes, which are intermediary metabolites of the catecholamines and related compounds,[2] may also be inhibited by this substance. This possibility was investigated in the guinea pig using normetanephrine, the O-methyl derivative of norepinephrine.

Normetanephrine-1-^{14}C, with a specific activity of 1·0 μc/μmole, was synthesized from vanillincyanohydrin-1-^{14}C by reduction with $LiAlH_4$.[3] Female guinea pigs weighing between 350 and 450 g were injected with 100 μg of this substrate and their urines collected during a 24 h period over $CHCl_3$. A portion of the combined urines was extracted with 4 vols. of alcohol–acetone (1 : 1) and the extract applied to a strip of Whatman no. 1 filter paper and developed overnight in *n*-butanol–acetic acid–water, (4 : 1 : 1). The dried chromatogram was scanned in a gas flow counter coupled with a recording device. The relations of the separated radioactive metabolites are shown in scan *A*, Fig. 1. The major

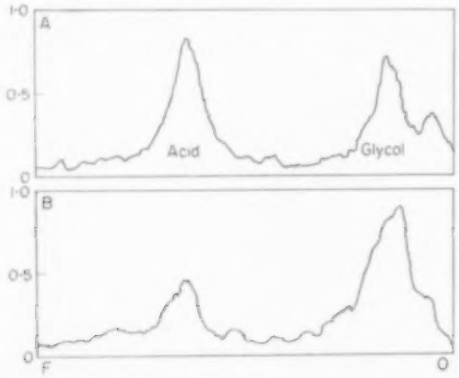

FIG. 1. Distribution and relative amounts of radioactive metabolites on the scanned chromatograms of urines from guinea pigs given normetanephrine-1-^{14}C. Scan *A*: untreated animals. Scan *B*: animals injected intraperitoneally with 200 mg of disulfiram per kg daily for 3 days prior to the administration of normetanephrine-1-^{14}C. Descending chromatography in *n*-butanol–acetic acid–water (4 : 1 : 1). *O* and *F*: origin and front.

* Disulfiram (tetraethylthiuram disulfide) was kindly supplied by Ayerst Laboratories.

radioactive metabolite at $R_f = 0{\cdot}65$ was identified as vanillylmandelic acid (VMA) by distribution coefficients and chromatographic behavior. The second major peak at $R_f = 0{\cdot}2$ represents primarily a compound which can be adsorbed onto an anion exchange resin (Dowex 1X2), but not a cation exchange resin (Dowex 50X16). This anionic compound had an R_f of 0·55 in *iso*propanol–7 N NH_3 (4 : 1). After incubation at 55 °C for 8 hr with sulfatase (Mylase P), a compound was obtained which had the R_f of 0·67 in both butanol–acetic acid and *iso*propanol-ammonia systems. A product with identical chromatographic properties was prepared by reduction of VMA (K & K Laboratories) with $LiAlH_4$. Axelrod *et al.*[4] have shown that 3-methoxy-4-hydroxyphenylglycol sulfate, a compound with similar properties, is a major metabolite of norepinephrine in the rat. The evidence suggests that the peak at R_f 0·2 represents the glycol sulfate. The remaining compounds closer to the origin are unidentified.

The effect of disulfiram on the metabolism of normetanephrine-1-^{14}C was determined in a comparable group of guinea pigs. Disulfiram was prepared as a suspension of 50 mg/ml of propylene glycol–water (1 : 1), and injected intraperitoneally daily for 3 days in a dose of 200 mg/kg. About 6 h after the last dose, 100 μg normetanephrine-1-^{14}C were injected intraperitoneally and the urines collected and processed as before. Chromatography of the urinary extract showed a substantial decrease in VMA relative to the total radioactivity in the urine (scan *B*, Fig. 1). A concomitant increase in the peak representing the glycol sulfate also appears to have taken place. This finding, confirmed by subsequent experiments, suggests that oxidation of the intermediate glycol aldehyde to VMA is inhibited by disulfiram and, as a consequence, formation of the corresponding alcohol is enhanced. This study has been extended to include preliminary experiments with other biologically active amines. In disulfiram-treated rats, significant quantities of tryptophol[5] and tyrosol can be detected in their urines after administration of tryptamine and tyramine. Evidently, disulfiram can inhibit the oxidation of several β-substituted acetaldehydes. This work was supported by a grant (M-1434) from the United States Public Health Service.

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ANNOUNCEMENTS

First International Pharmacological Meeting

This meeting will be held in Stockholm, Sweden, on August 22–25, 1961, under the auspices of the Section of Pharmacology of the International Union of Physiological Sciences.

The programme is designed to promote attendance by members of the International Union of Biochemistry, visiting the Fifth International Congress of Biochemistry.

It has been decided to restrict the scientific programme to topics under the general heading of "Mode of action of drugs":

Pharmacological control of release of hormones, including antidiabetic drugs.
Effects of drugs on synthesis and mobilization of lipids.
New aspects of cardic pharmacology.
Drugs and membranes.
Methods for the study of pharmacological effects at the cellular and subcellular levels.
Metabolic factors controlling duration of drug action.
Pharmacological analysis of central nervous action.

The convention will provide prominent workers in the respective fields to deliver introductory lectures. In addition to these brief communications reporting original research will be accepted insofar as facilities permit, and will be scheduled after the introductory lectures.

For all inquiries, and to obtain this and subsequent pamphlets, please write to the **Secretariat of the First International Pharmacological Meeting, Secretary General, Dr. Arvid Wretlind, Karolinska Institutet, Stockholm 60, Sweden.**

Editors' and Publishers' Note

During 1960 it is intended to publish a total of between 1000 and 1200 pages. In addition to the normal contributed papers, Biochemical Pharmacology will publish the proceedings of symposia on topics of direct interest to Biochemical Pharmacology.

Papers to be published in future issues

TIBOR ZSOLNAI: Versuche zur Entdeckung neuer Fungistatika—I. Phenol-derivative.

T. MASURAT, S. M. GREENBERG, E. G. RICE, J. F. HERNDON and E. J. VAN LOON: The action of chlorpromazine on yeast hexokinase.

B. W. FOX, A. W. CRAIG and H. JACKSON: The comparative metabolism of myleran-^{35}S in the rat, mouse and rabbit

BO FREDHOLM, BERTIL HÖGBERG and BÖRJE UVNÄS: Role of phospholipase A and C in mast cell disruption induced by non-purified *Clostridium welchi* toxin.

NORMAN H. CAREY and H. GEORGE MANDEL: The metabolism of 6-mercaptopurine by *Bacillus cereus*.

MARJORIE NEWELL, MARY ARGUS and F. E. RAY: Routes of metabolism of [^{36}Cl] ring-substituted monochloroacetanilides.

PRISCA L. HÁGONY and M. JÁKY: Changes in the lipid composition of tumour and hepatic tissues of rats bearing güerin carcinoma following treatment with the nitrogen mustard degranol.

D. W. VISSER, D. M. FRISCH and BESSIE HUANG: Synthesis of 5-chlorodeoxyuridine and a comparative study of 5-halodeoxyuridines in *E. coli*.

O. A. TROWELL: The cytocidal action of mitotic poisons on lymphocytes *in vitro*.

THOMAS G. SCHARFF and WILMITCH C. MAUPIN: Relationship of cell structure to metabolism—I. Correlation of the metabolic effects of benzalkonium chloride with its membrane effects in yeast.

H. BALZER, P. HOLTZ and D. PALM: On the mechanism of the convulsant effect of hydrazides in mice.

K. J. CHILD, PATRICIA SUTHERLAND and E. G. TOMICH: The effects of drugs on barbital anaesthesia in mice.

E. G. ERDÖS, C. R. DEBAY and M. P. WESTERMAN: Arylsterases in blood: Effect of calcium and inhibitors.

PARKHURST A. SHORE and VICTOR H. COHN, JR.: Comparative effects of monoamine oxidase inhibitors on monoamine oxidase and diamine oxidase.

LIBOR SLECHTA: Studies on the mode of action of psicofuranine (6-amino-9-D-psicofuranosylpurine).

A. C. M. PAIVA and THEREZINHA B. PAIVA: The importance of the N-terminal end of angiotensin II for its pressor and oxytocic activities.

L. A. ELSON: Haematological effects of homologous series of dichloroethylarylamines (aromatic nitrogen mustards).

T. A. CONNORS, L. A. ELSON, A. HADDOW and W. C. J. ROSS: The pharmacology and tumour growth inhibitory activity of 1-aminocyclopentane-1-carboxylic acid and related compounds.

W. JACOBSON and I. A. B. CATHIE: The inactivation of folic acid antagonists by normal and leukaemic cells

W. JACOBSON and I. A. B. CATHIE: The nature of aminopterin inactivated by normal and leukaemic tissues.

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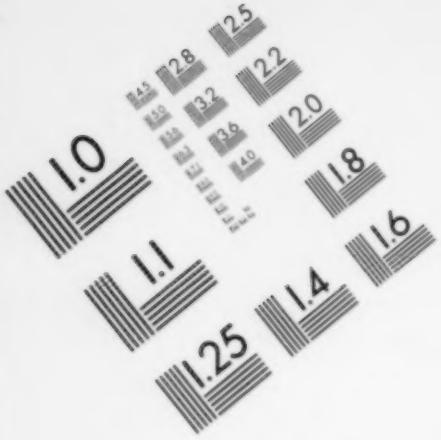

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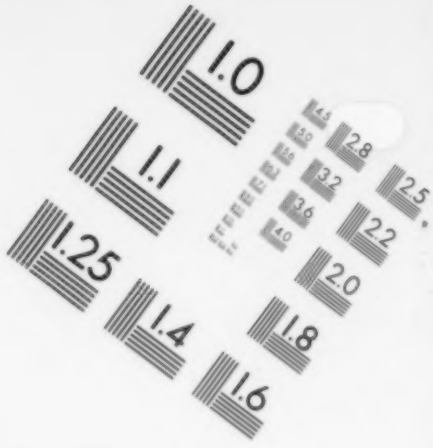

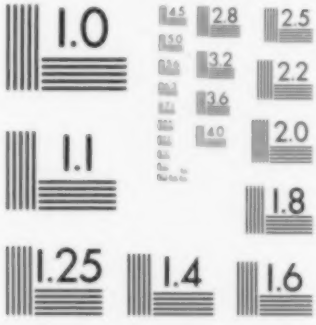

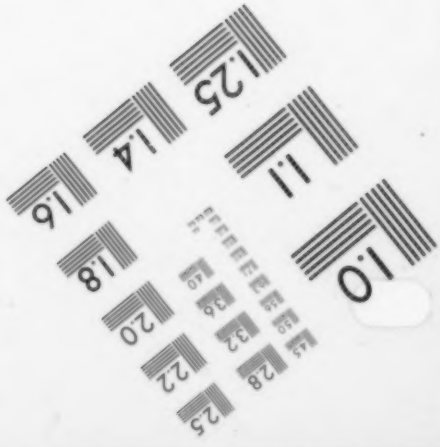

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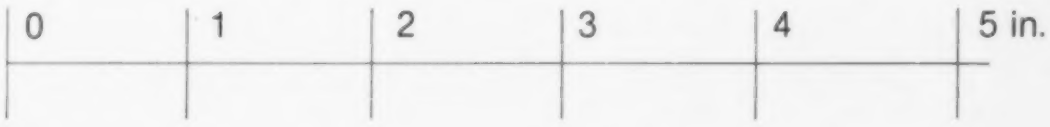

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